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**Understanding at the molecular level
the consequences of defective ATR
signalling in humans**

By

Diana Alcântara

A thesis submitted to the University of Sussex for the degree of Doctor
of Philosophy (DPhil)

December 2012

DECLARATION

I hereby declare that this thesis has not been and will not be submitted in whole or in part to another University for the award of any other degree.

Signed

Diana R. R. Alcântara

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This thesis is dedicated to Luis Ralha

University of Sussex

Diana R. R. Alcantara

DPhil Biochemistry

Understanding at the molecular level the
consequences of defective ATR signalling in
humans

SUMMARY

Ataxia telangiectasia and Rad3-related (ATR) is a central regulator of the mammalian DNA damage response. ATR is essential for survival, but hypomorphic mutations in ATR are associated with a subset of Seckel syndrome (ATR-S), a human condition characterised by microcephaly and severe growth retardation. I used patient-derived cell lines, as well as siRNA ATR inhibition to evaluate the impact of ATR deficiency in skeletal development (osteogenesis and chondrogenesis). The expression of chondroinduction-specific markers was also assessed and found impaired in ATR-S and PCNT-S patient-derived primary fibroblasts. I also found signs of insulin/PI3K/mTOR signalling deregulation in both ATR as well as PCNT deficient cells. A checkpoint defect in a disorder with a primary defect in mTOR pathway function, Donohue Syndrome, was also uncovered, establishing a link between defects in insulin/mTOR signalling with deregulation of the DNA damage response/checkpoint machinery, possibly via the glycogen synthase kinase 3 β , GSK3 β , in human patient cells. I characterized cellular functional aspects of a novel microcephalic disorder, Microcephaly-Capillary Malformation Syndrome (MIC-CAP), caused by mutations in the endosome-associated deubiquitinase enzyme STAMBP. MIC-CAP cells exhibit compromised RAS-MAPK and PI3K-AKT pathway signalling, and defective ATR-dependent DDR, a common feature of microcephalic disorders. I also characterized aspects of PI3K-AKT signalling in two megalencephaly disorders harbouring novel mutations in core components of this pathway. Finally, I evaluated aspects of mTOR pathway function in cells from a genomic disorder caused by copy-number variation of 1q21.1.

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ABBREVIATIONS

A-T	ataxia-telangiectasia
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia mutated and Rad3-related
ATRIP	ATR interacting protein
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CHX	cycloheximide
DAPI	4'-diamino-2-phenylindole
DNA-PK	DNA-dependent protein kinase
DSB	double strand DNA break
FA	Fanconi Anaemia
FCS	foetal calf serum
HR	homologous recombination
hrs	hours
HU	hydroxyurea
IF	immunofluorescence
IR	ionising radiation
LCL	lymphoblastoid cell line
MCPH	primary microcephaly
MEF	mouse embryonic fibroblast
MRN	Mre11-Rad50-Nbs1
NBS	Nijmegen Breakage syndrome
PBS	phosphate buffered saline
PIKK	phosphatidylinositol 3-kinase like kinase
RNAi	RNA interference
ROS	reactive oxygen species
RPA	replication protein A
SD	standard deviation

siRNA	short-interfering RNA
SSB	single strand DNA break
ssDNA	single-stranded DNA
TBS	tris-buffered saline
Ub	ubiquitin
UNT	untreated
UV	ultra-violet
WB	western blot
WT	wild type ; clinically normal individual

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Chapter One

Introduction

Introduction

Perturbation of organism size, growth and development, and the mechanisms that regulate it, are of key understanding in human disease. *Primordial dwarfism* is a term that designates a group of human disorders displaying extreme global growth failure, which usually also manifests with proportionally reduced (or even smaller) head size (microcephaly) (McKusick 1955; Rauch et al. 2008). Severe microcephaly is defined as occipitofrontal circumference (OFC) 3 standard deviations or more below the norm for the age and gender (Verloes et al. 2004; Woods 2004). Microcephalic primordial dwarfism (MPD) is then a group of autosomal recessive conditions (subtypes described in 1.2) that couples acute intrauterine and postnatal growth retardation with severe microcephaly and mental retardation.

Organ and organismal size is effectively determined by cell number and volume (Conlon and Raff 1999). Some of the mechanisms that regulate organism growth will also be discussed further below.

In this thesis, I will present several distinct though interwoven stories concerning the interaction between aspects of the DNA damage response (DDR), cell cycle checkpoint control and mTOR pathway function using cell lines from various human disorders including MPD, megalencephaly disorders and metabolic disorders. Collectively, this data highlights the importance of the functional crosstalk between these distinct pathways for normal human development. But first, I will provide some background on the origin of microcephaly and related human disorders to overview their aetiology and molecular pathologies

1.1 Mammalian brain development

Reduced head size is the reflection of reduced cerebral cortex volume, likely linked to impaired neural cell proliferation and/or increased apoptosis during early embryogenesis. Several classes of neuroprogenitor cells undergo rapid and sustained cellular proliferation, migration and differentiation at the foetal neural tube: neuroepithelial cells (NE), radial-glia cells and basal (or intermediate) progenitors (Thornton et al. 2009). The totality of neurons produced during the development of the CNS is determined by type and number of cell divisions undergone by these progenitor cells. NE cells are the original progenitor cells, extending from the basal

lamina to the apical ventricular layer. NE cells divide symmetrically laterally to generate more progenitor cells, or asymmetrically to originate the other “downstream” types of progenitor cells. Both symmetric and asymmetric divisions of the all these types of progenitor cells give rise to the neurons (Huttner et al. 2005; Götz et al. 2005).

NE cell are polarized along the apical-basal axis in the neural epithelium, with the nucleus migrating between the surfaces. S-phase occurs with the nucleus located near the basal lamina, which then migrates towards the apical surface where mitosis occurs. The apical-basal polarity in the neuroepithelium is important for the establishment of the cleavage plan for symmetric and asymmetric cell divisions (Huttner et al. 2005; O'Driscoll et al. 2008).

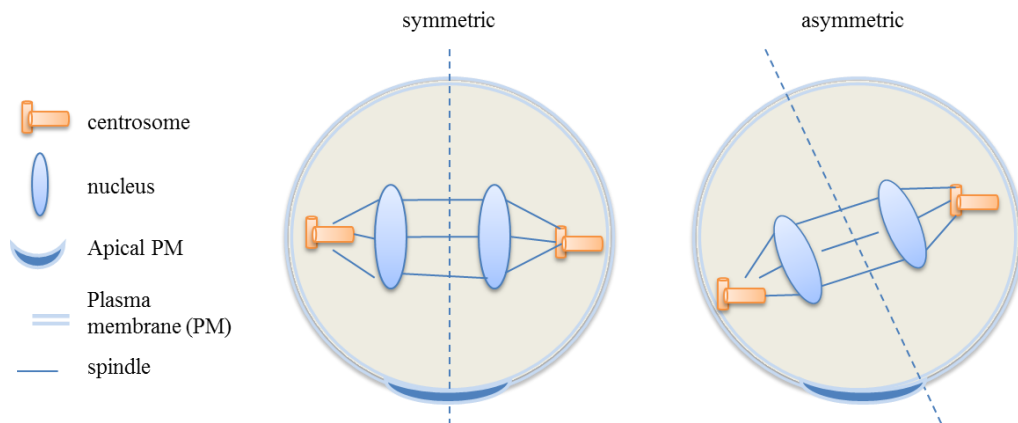


Figure 1.1 Symmetric vs asymmetric progenitor cell division.

Orientation of the mitotic spindle is perpendicular to the apical-basal axis during symmetric cell division, with the resulting cleavage plan intersecting the apical PM. A deviation in the orientation of the mitotic spindle gives rise to an asymmetric cell division, with only one of the daughter cells inheriting the apical PM region.

The apical plasma membrane of NE cells is attached by adherens junctions to the apical surface. Asymmetric cell division would result in apical cells constituents being inherited by one daughter cells, and basal constituents by the other, giving rise to one other progenitor cell and a neuron. An unequal inheritance of polarized cell constituents such as the apical PM means that both symmetric as well as asymmetric cell divisions can derive from a perpendicular cleavage plan. The correct orientation of the mitotic spindle is therefore crucial during neurogenesis, and highly regulated. The number of rounds of symmetric proliferative divisions undergone by NE cells

determines the ultimate number of neurons. As neurogenesis progresses, increased asymmetric divisions means that only one daughter cell inherits the apical PM and remains a NE cell, while the other becomes either a basal progenitor, a radial-glia cell or a neuron (S. Zhang et al. 2007; Götz et al. 2005; Huttner et al. 2005).

Additionally, cell cycle length plays a role in proliferative *vs* differentiating cell divisions. Longer cell cycles have been associated with differentiating divisions, while for example shortening of G1 phase has been linked to an increase in proliferative divisions (O'Driscoll et al. 2008). Reduced neuroprogenitor proliferative capacity is a likely cause of microcephaly.

1.1.1 Primary microcephaly

Reduction of brain size in MPD is similar to that observed in autosomal recessive primary microcephaly (MCPH). MCPH patients exhibit significantly prenatal brain size reduction, but not usually associated with pronounced alterations in the brain architecture (Poulton et al. 2011). This suggests that defective neurogenic proliferation could underlie the microcephaly seen in MCPH. These patients have mild to moderate mental retardation, but do not share other clinical features typical of MPD, such as severe growth retardation and skeletal abnormalities (Woods 2004).

Several susceptibility *loci* have been linked to primary microcephaly to date (Table 1), and all the MCPH proteins identified so far associate with the centrosome (Thornton et al. 2009).

Table 1.1 Primary microcephaly loci.

(adapted from Thornton and Woods 2009)

<i>locus</i>	OMIM	Chromosome location	Gene
MCPH1	251200	8p23	<i>Microcephalin (MCPH1)</i>
MCPH2	604317	19q12	<i>WDR62</i>
MCPH3	604804	9q33.3	<i>CDK5RAP2/CEP215</i>
MCPH4	604321	15q21.1	<i>CEP152</i>
MCPH5	608716	1q31	<i>ASPM</i>
MCPH6	608393	13q12.2	<i>CENPJ</i>
MCPH7	612703	1p32	<i>STIL</i>

MCPH1 encodes a BRCT domain containing protein that functions in cell cycle progression and also in the DNA damage response, both mechanisms that are known to affect neurogenesis. MCPH1 is required for the recruitment of DDR proteins (Doxsey, et al. 2005; X. Wu et al. 2009), and a defective G2/M checkpoint activation has also been observed in MCPH1 patient derived cells (Alderton et al. 2006). Studies have shown that MCPH1 and PCNT are responsible for the recruitment of the checkpoint kinase CHK1 to the centrosome, and loss of MCPH1 would result in premature entry into mitosis (Tibelius et al. 2009). ASPM (Abnormal Spindle Microcephaly Associated) localizes to mitotic spindle poles and is involved in spindle organization/positioning (Higgins et al. 2010), being highly expressed during the period of rapid proliferative expansion of the cerebral cortex in the mouse (Pulvers et al. 2010). CENPJ is required for centriole biogenesis, and loss of centrioles results in spindle malformation and defective chromosome segregation (Bond et al. 2005). The study of MCPH genes has highlighted the importance of centrosome function and spindle formation in the development of microcephaly, with impacts on other mechanisms such as defects in the DDR.

1.2 MPD disorders

Microcephalic primordial dwarfism encompasses a range of single-gene autosomal recessive disorders, which include Seckel-syndrome (SS), microcephalic osteodysplastic primordial dwarfism type II (MOPDII) and Meier-Gorlin syndrome (MGS). Disproportionally reduced brain size distinguishes MPD from other forms of dwarfism.

1.2.1 Seckel syndrome

A group of patients was described by Helmut Seckel in 1960 as “bird-headed” or “microcephalic” dwarfs, due to their characteristic facial features (Figure 1.2) (Q. Wang et al. 2004; Seckel 1960). The term Seckel syndrome (OMIM 210600) is now used to encompass unspecified and heterogeneous forms of microcephalic primordial dwarfism characterized by intrauterine and postnatal growth retardation, proportionate dwarfism, severe microcephaly (OFC -4 to -14SD)

and mental retardation, in addition to the typical facial features (Knoblich 2010; Majewski et al. 1982). Abnormalities associated with SS also include extremely delayed skeletal maturation with retarded ossification resulting in skeletal abnormalities (Jackman et al. 2003; Børglum et al. 2001); *Acanthosis nigricans*, a hyperpigmentation of the skin commonly caused by insulin resistance has also been reported in SS. The association of SS with cancer is not clear, but some cases have been reported (Faivre et al. 2002; Butler et al. 1987).

Consistent with the heterogeneity of the clinical features, a number of genomic susceptibility loci associated with SS have been identified to date.

The first locus was mapped to chromosome 3q22.1-q24 (*Sckl1*) where the disease was found to be caused by a mutation in the gene encoding the apical DDR protein ataxia-telangiectasia and RAD3-related (ATR) (Diviani et al. 2000; O'Driscoll et al. 2003). The single synonymous 2101A→G transition was shown to affect splicing efficiency resulting in low levels of ATR protein (Figure 1.2). Patient cell lines exhibited defects in the ATR-dependent DDR, such as impaired H2AX phosphorylation in response to UV irradiation (J. Kim et al. 2008; O'Driscoll et al. 2003). Interestingly, microcephalin (*MCPH1*), the first causative gene linked with primary microcephaly, is also associated with defective ATR pathway function (Alderton et al. 2006).

Mutations in the gene coding for the ATR interacting protein, *ATRIP* (ataxia-telangiectasia and RAD3-related-interacting protein), phenocopy ATR loss (Tibelius et al. 2009; Ogi et al. 2012). Cells from an *ATRIP*-mutated SS patient also exhibit reduced ATR expression (due to lack of ATR/ATRIP co-stabilization) and impaired ATR-dependent DDR (S. Zhang et al. 2007; Ogi et al. 2012).

Other SS-causing genetic defects identified include *CENPJ* and *CEP152* (Table 1.2). *CENPJ* (Centromere protein J; also known as CPAP) is a centrosomal protein with roles in spindle assembly/disassembly and centriole duplication, important for maintenance of centrosome integrity and cell cycle control (Chen et al. 2006). *CENPJ* interacts with Pericentrin (PCNT), mutated in the SS-related MOPDII. *CEP152* (centrosomal protein 152) is a scaffolding protein, component of the pericentriolar material, involved in recruitment of proteins to the centrosome. *CEP152* interacts also with the Polo-like kinase, PLK4 and *CENPJ*. *CEP152* mutations have also been observed in primary microcephaly (Guernsey et al. 2010), as well as mutations in *CEP63* (Sir et al. 2011). *CEP63* is required for CDK1

localization to the centrosome (Löffler et al. 2011). *Cep 63^{-/-}* in DT40 cells results in mitotic spindle defects (Sir et al. 2011).

Mutations in CtIP (CTBP-interacting protein), a protein involved in end-resection at DSBs have also been identified in SS (Qvist et al. 2011). CtIP has an established role in DNA repair (Delaval and Doxsey 2010; Sartori et al. 2007), interacting with the MRN complex at sites of DSBs. Loss of CtIP function in processing DSBs leads to a reduction of RPA-coated ssDNA regions and consequently compromised ATR activation (Qvist et al. 2011). A novel association between Seckel syndrome and the centrosomal protein ninein (NIN) was recently found (Dauber et al. 2012).

ATR knockdown is embryonic lethal. A mouse model of SS has been generated, incorporating the original Seckel syndrome *ATR*-splicing mutation (O'Driscoll et al. 2003) (Figure 1.2). The ATR-SS mouse exhibits all the hallmarks of SS, including the severe growth retardation and microcephaly with micrognathia (Murga et al. 2009). Elevated apoptosis was observed during embryogenesis in this model, as a consequence of increased replicative stress-induced DNA damage that results from ATR depletion (Murga et al. 2009). Notably, the authors noted a suppressed Insulin-like Growth Factor-1/Growth hormone axis in the ATR-SS mouse, which could also have an impact on the growth retardation phenotype. Moreover, conditional *Atr* knockdown in adult mice results in depletion of a high percentage of proliferating cells and an age-related phenotype (Ruzankina et al. 2007).

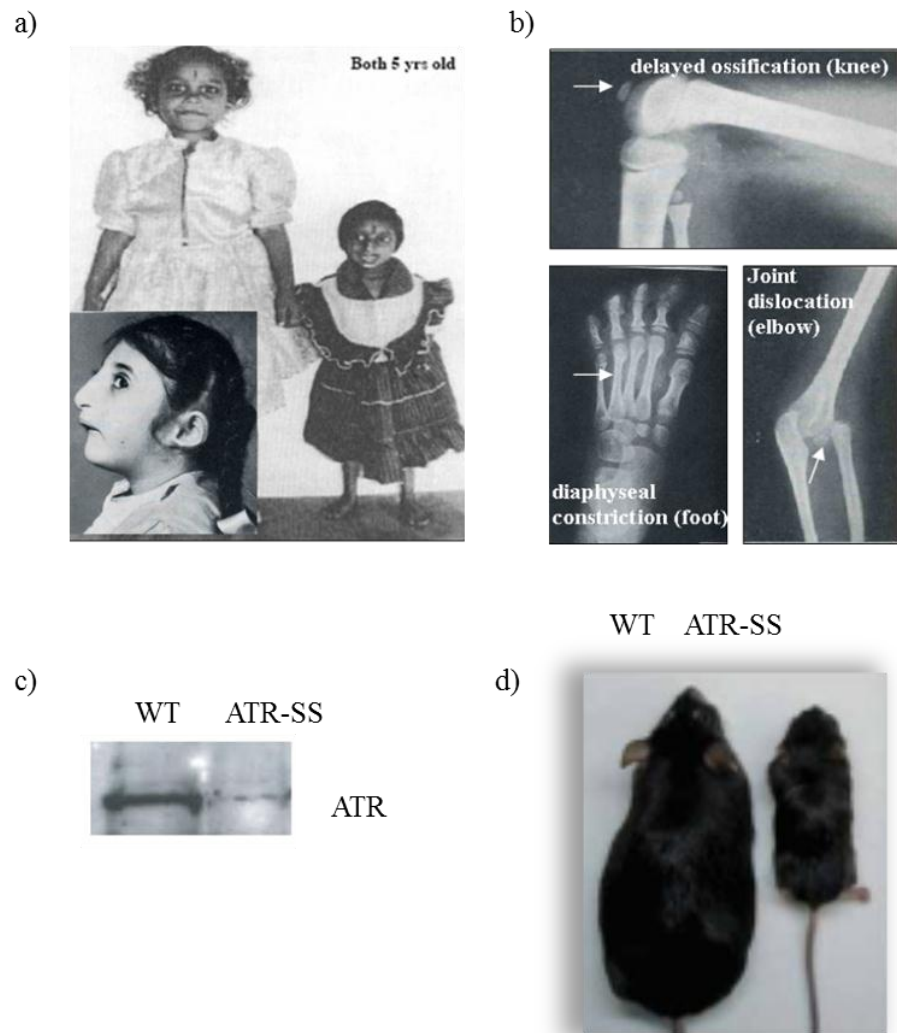


Figure 1.2 ATR-Seckel Syndrome.

a) SS patients show markedly reduced stature and microcephaly, with characteristic 'bird-headed' profile (inset) with receding forehead, micrognathia, prominent teeth, microtia (small ears). **b)** pronounced skeletal abnormalities are also a feature of SS. **c)** western blot showing the reduced ATR protein expression in ATR-SS patient cells (O'Driscoll et al. 2003). **d)** "humanized" ATR-Seckel mouse, incorporating the human ATR hypomorphic mutation, recapitulates SS clinical features (X. Yang et al. 2008; Murga et al. 2009).

1.2.2 MOPDII

Microcephalic Osteodysplastic Primordial Dwarfism-type II (MOPDII) is a defined form of MPD, clinically similar to SS but regarded as a separate entity (Majewski and Goecke 1982). Distinguishing features include disproportionately shorter limbs, distinct skeletal features and microcephaly that progresses postnatally (Hall et al. 2004), accompanied by milder mental retardation. A risk of neurovascular problems (moyamoya disease) has been associated with MOPDII (Bober et al. 2010), with the life-threatening development of brain aneurysms and seizures (J. G. Hall et al. 2004). Additionally, MOPDII individuals develop insulin resistance during childhood (with *acanthosis nigricans*) and type II diabetes (Huang-Doran et al. 2011).

Mutations in the gene encoding pericentrin (*PCNT*) - a key structural centrosomal protein involved in spindle assembly during mitosis - have been identified in MOPDII (Griffith et al. 2008). *PCNT* mutations have been previously associated with patients clinically diagnosed as Seckel syndrome. Over- and mis-diagnosis of SS has led to some confusion in the literature (Thompson and Pembrey 1985), and the current opinion is now that the *PCNT*-Seckel patients reported are in fact MOPDII patients. For sake of simplicity I will use the term *PCNT*-S in this thesis to refer to *PCNT*-mutated MDP. Importantly, cells from *PCNT*-mutated Seckel syndrome/MOPDII patients are also defective in ATR-dependent DNA damage signaling, and *PCNT* knockdown results in compromised ATR-dependent checkpoint activation (Griffith et al. 2008).

1.2.3 Meier-Gorlin syndrome

Meier-gorlin syndrome (MGS), also known as “ear, patella and short stature” syndrome, is defined by absent or hypoplastic patellae (knee caps) and marked microtia (small ears) (Bongers et al. 2001). MGS patients have microcephaly with mild intellectual disability, growth failure and skeletal abnormalities (Faqeih, Sakati, and Teebi 2005). Mutations in the pre-replication complex components have recently been found in a group of MGS patients. Mutated MGS genes include *ORC1*, *ORC4*, *ORC6*, *CDC6* and *CDT1*. These mutations suggest a link between defective DNA replication (in this case origin licensing) and MPD. Interestingly, *ORC1* defective

MGS-patient derived cell lines also exhibit defects in the ATR-dependent signalling response to DNA damage (Sarah Walker, GDSC).

Table 1.2 Genes mutated in MPD.

Gene	OMIM	MPD	Gene function
<i>ATR; ATRIP</i>	210600	Seckel	Master regulator of DDR
<i>CENPJ/CPAP</i>	613676	Seckel	Centriole biogenesis
<i>CEP152</i>	613823	Seckel	Centriole biogenesis; genome stability
<i>CEP63</i>	614728	Seckel	Centrosome function
<i>RBBP8(CtIP)</i>	606744	Seckel	DNA DSB resection
<i>NIN</i>	614851	Seckel	Centrosome function; microtubule organization
<i>PCNT</i>	210720	MOPDII	Component of pericentriolar material; scaffold for signaling molecules
<i>ORC1,ORC4,ORC6, CTD1, CDC6</i>	224690 613800 613800 613804 613805	MGS	Components of pre-replication complex; licensing of replication origins
<i>IGF1</i>		IGF1 deficiency	Endocrine/paracrine growth hormone
<i>U4atac</i>	210710	MOPDI	Spliceosome component

Finally, Netchine and colleagues recently described an extremely rare syndrome caused by homozygous mutations in *IGF-1* (Insulin-like Growth Factor-1) (Netchine et al. 2011). The clinical presentation of *IGF-1* mutated patients is extremely similar to other MPD types, including intrauterine and postnatal growth retardation, microcephaly and mental retardation. Sensorial deafness is a specific trait of this disorder. Additionally, insulin resistance, partial gonadal dysfunction and osteoporosis have also been observed in IGF-1 deficient patients (Netchine et al. 2011). This supports a role for IGF-1 in neuronal development and growth. The authors suggest that recombinant IGF-1 therapy is likely beneficial to the management of this disorder. There are indications that growth hormone (GH) therapy in Seckel syndrome children had a positive effect on final height, associated with increased serum-levels of insulin-like growth factors (Guertin et al. 2006; Birkebaek et al. 2011).

The intersection between ATR signaling and centrosomal proteins appears to be a recurrent theme in MDP. Overall, MPD-causative mutations seem to affect the efficiency of cell cycle progression, resulting in reduced cell number generated during development, in particular during neurogenesis, and consequently giving rise to small brain and body size.

1.3 DNA damage response

Microcephaly is a common feature of many disorders associated with defective DNA damage response (DDR) (O'Driscoll and Jeggo 2008). An efficient DDR is therefore crucial during normal brain development.

Maintenance of genomic stability and the faithful transmission of the information held within the DNA during cellular division is an essential function carried out by the cell cycle and the DDR machineries. Cells are susceptible to numerous agents that can cause damage to the DNA, both endogenous as well as exogenous. Reactive oxygen species (ROS), generated during normal metabolism, induce base and sugar lesions that can result in single stranded and double stranded DNA breaks (SSBs; DSBs) (Cooke et al. 2003; Cadet et al. 2003). DNA damage resulting from hydrolysis and methylation can also occur during normal cellular processes (DeBont 2004). DNA DSBs also occur during V(D)J recombination in developing B and T cells (Jeggo et al. 1995). Depending on cell cycle context (i.e. the presence of a homologous sister) DSBs are repaired by non-homologous end-joining (NHEJ) in mammalian cells, and/or homologous recombination (HR) (Sargent et al. 1997). External agents such as ionizing radiation (IR) directly induce complex breaks in the genome, including DSBs. Additionally, exposure to UV radiation results in bulky lesions, including pyrimidine dimers, whose processing generates regions of ssDNA. UV-induced dimers also perturb the normal progression of replication and transcription complexes. Finally, chemicals such as the Ribonucleotide Reductase inhibitor hydroxyurea (HU), which causes replication fork arrest, can also result in DNA breaks (Sakano et al. 2001).

DNA damage is a major source of mutagenesis, carcinogenesis and ageing. As such, cells have evolved complex and highly regulated mechanisms of detecting DNA damage, and either elicit the repair response, involving cell cycle arrest; or alternatively activate the programmed cell death pathway, in order to prevent accumulation of mutations and proliferation of damaged cells.

The DDR is the coordination of DNA damage sensors, signal transducers, mediators and effector proteins (Harper et al. 2007; Zhou et al. 2000).

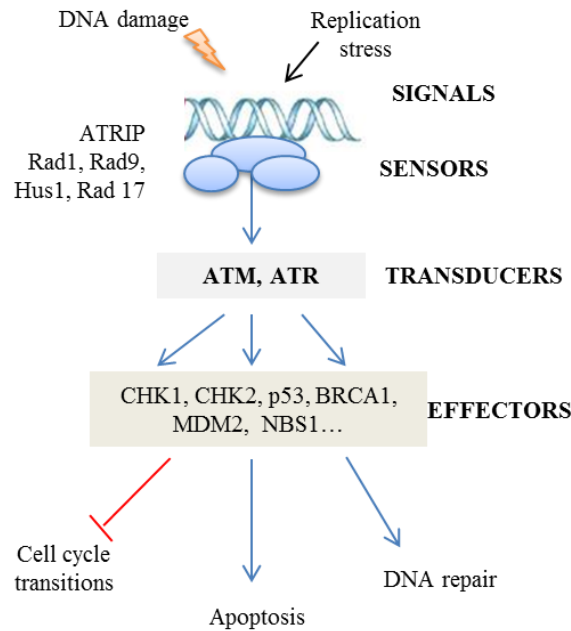


Figure 1.3 General outline of the DNA damage response signal-transduction pathway. DNA damage is detected by sensor proteins which then activate ATM/ATR. These transducers in turn signal to downstream effectors leading to either cell cycle arrest or apoptosis.

The principal regulators of the DDR are the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Ataxia telangiectasia mutated (ATM) and Ataxia telangiectasia and Rad3 related (ATR), which are members of the phosphatidylinositol 3-kinase like kinases (PIKKs) family of protein kinases. Other PIKKs include the mammalian target of rapamycin (mTOR), the suppressor of morphogenesis in genitalia (SMG1) and transformation/transcription domain-associated protein (TTRAP) (Lempiäinen et al. 2009). The ATR and ATM kinases preferentially phosphorylate substrates with a consensus SQ/TQ motif, where a serine or threonine residue is followed by a glutamine (Shechter et al. 2004a).

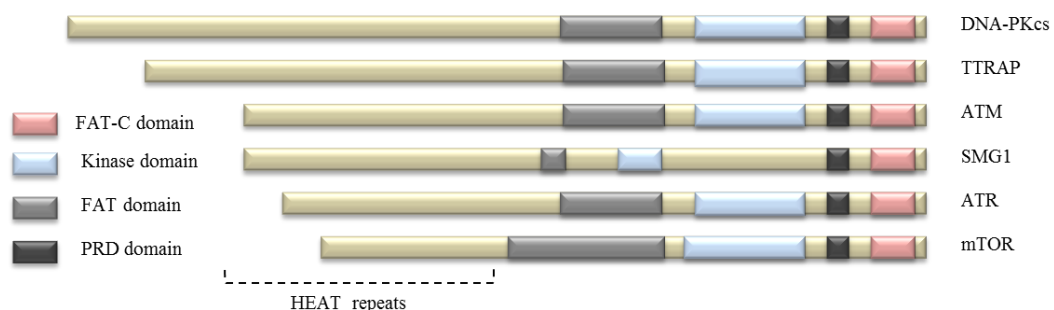


Figure 1.4 The PIKK family of kinases. The FATC (FRAP-ATM-TTRAP), SQ/TQ-directed protein kinase, PIKK-regulatory (PRD) and FAT-C (FAT-C terminal) domains of homology are indicated. The amino terminal region contains multiple HEAT (Huntington, Elongation factor 3, A subunit of protein phosphatase, TOR1) repeats (adapted from Derheimer et al. 2010).

The ATM and DNA-PKcs kinases are activated by DSBs, resulting for example from IR-induced damage. DNA-PKcs associates with Ku70-Ku80, forming the DNA-PK complex, which allows its recruitment to sites of DSB and DNA-PKcs activation. DNA-PKcs is important for NHEJ, a main DSB repair pathway (Burma and Chen 2004).

ATM activation occurs early following DSB formation, via interaction with the Mre11-Rad50-Nbs1 (MRN) complex (Bakkenist and Kastan 2003), which is the initial sensor of the DNA damage. ATM is a primary transducer of the cellular response to DSBs, and activation of ATM-dependent cell cycle checkpoints can mediate cell cycle arrest, via some of the key ATM substrates: the checkpoint kinase 2 (CHK2), p53, p21. ATM is mutated in the neurodegenerative disorder Ataxia telangiectasia (A-T; OMIM 208900), which is associated with cancer predisposition (O'Driscoll and Jeggo 2006). A-T patient cells are sensitive to killing by IR and agents that induce DSBs, and exhibit defective cell cycle checkpoint activation (Riballo et al. 2004).

1.4 ATR activation/ ATR-dependent signaling

ATR, together with ATM, constitute the two key apical regulators of the mammalian DDR and cell cycle checkpoint pathways.

ATR is activated by regions of single-stranded DNA (ssDNA), generated during normal replication or replication-fork stalling in S-phase, or as an intermediate in DNA repair pathways like the nucleotide excision repair (NER) and homologous recombination (HR) pathways (Byun et al. 2005). ATR can also be activated by persistent DSBs in late S-phase or G2, in an ATM-dependent manner, following resection to generate single-stranded regions of DNA (de Klein et al. 2000; Ward, Minn, and Chen 2004; Brown and Baltimore 2000; Byun et al. 2005).

ATR exists in the cells in a stable complex with the ATR-interacting protein (ATRIP), and the expression of each protein is dependent on the other (Lawlor et al. 2002; Cortez et al. 2001). The structure that effectively activates ATR is a region of RPA (Replication-protein A)-coated ssDNA, next to a region of dsDNA with a 5' end (Chalhoub et al. 2009; Cimprich and Cortez 2008). These structures are formed

normally during normal replication, or as a consequence of replication fork stalling (Zou et al. 2003). RPA-coated ssDNA is also an intermediate of repair pathways such as NER, and therefore ATR-dependent signaling is not just required in replicating cells but throughout the cell cycle as part of the DDR (O'Driscoll et al. 2003). The heterotrimeric RPA complex is composed of three subunits (RPA1-3) and promotes ssDNA stabilization, with important roles during replication as well as the assembly of signaling complexes onto sites of DNA damage. ATRIP is recruited to single stranded regions of DNA through its affinity for RPA, thus localizing ATR to sites of DNA damage/replication fork stalling. UV radiation elicits the NER pathway to remove the damage caused by the pyrimidine dimers. This results in the formation of RPA-coated ssDNA intermediates during the repair process, which activate ATR signaling. ATR-deficient cells are therefore sensitive to UV and replication inhibitors (O'Driscoll et al. 2003). ATR can also be activated by IR in an ATM dependent manner.

Chromatin-bound RAD9/RAD1/HUS1 (9-1-1 complex), recruited to sites of DNA damage under regulation of the RAD17/RFC2-5 complex (RSR), facilitates ATR activation and ATR-mediated substrate phosphorylation (Bermudez et al. 2003; You et al. 2002) (Figure 1.5).

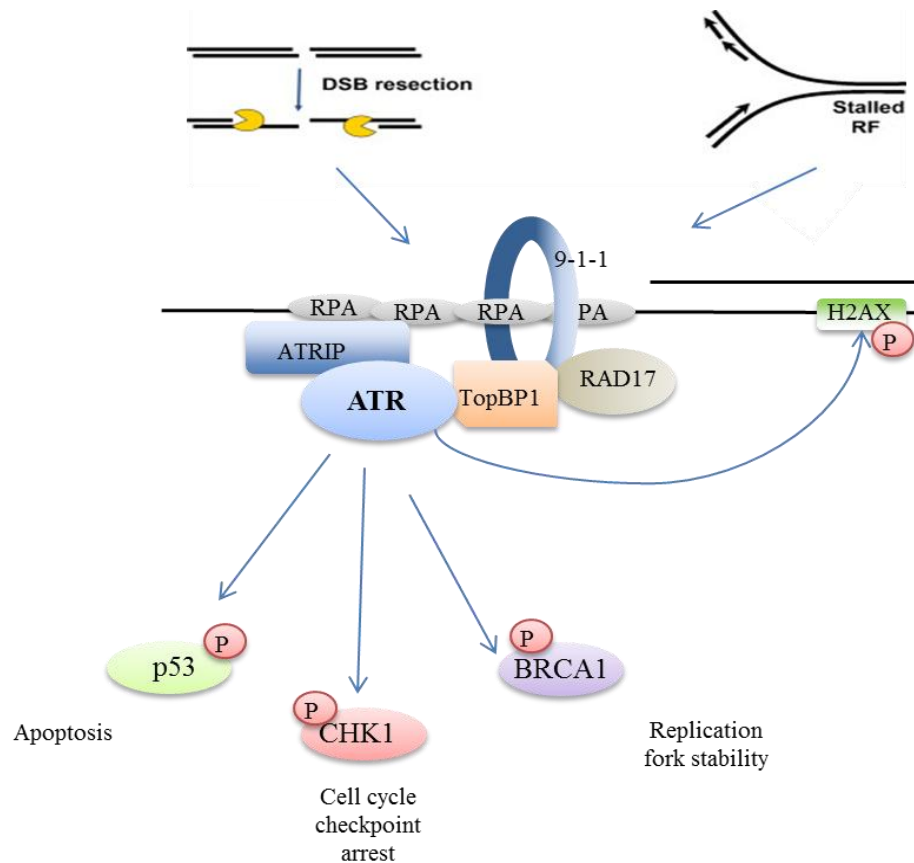


Figure 1.5 Model for ATR recruitment and activation. ssDNA (generated during DNA synthesis; or at resected double-strand DNA breaks for example), rapidly becomes coated with the stabilizing protein RPA, providing the signal that initiates an ATR-dependent response. RPA recruits ATRIP, which exists in a complex with ATR, localizing the kinase to the sites of DNA lesion. Additionally, RPA recruits Rad17, which loads the 9-1-1 complex. This steps are important to then recruit TopBP1, which activates ATR.

Recruitment of the RAD17/RFC2-5 complex to chromatin at DNA ends and junctions of ssDNA and dsDNA, is dependent on RPA, and independent of the ATR-ATRIP complex (Planchon et al. 2008; Zou et al.2002). Other factors that have been implicated in promoting activation of the ATR-ATRIP complex include the MRN complex and TopBP1. DNA topoisomerase II binding protein 1 (TopBP1) is recruited to sites of DNA damage via interaction with RAD9 (Martin et al. 2008; Greer et al. 2003), and its interaction with ATR-ATRIP is thought to stimulate ATR activation (Puc et al. 2005; Mordes et al. 2008). TopBP1 promotes ATR autophosphorylation at Threonine 1989, thought to mediate full activation of the ATR kinase (S. Liu et al. 2011).

However, association of ATR-ATRIP to chromatin is not absolutely essential for ATR signaling. Disruption of the RPA and DNA-binding domains in ATRIP mutants did not impede ATR-mediated CHK1 phosphorylation in *X. laevis* (Ball et al.2005).

Both ATR and ATM respond to DNA damage sensors by phosphorylating an array of independent as well as overlapping substrates that coordinate cell cycle progression with DNA repair, chromatin remodeling, or apoptosis. This implies there is a level of functional redundancy between the kinases. However, unlike ATM, ATR is absolutely required for cell viability. *Atr* knockout in mice results in early (pregastrulation) embryonic death, whereas *Atr*^{+/-} mice display a decrease in longevity, with an increase in the incidence of tumors, including histiocytic sarcomas, lymphomas and sebaceous gland adenomas (Brown et al. 2000). Accordingly, chromosomal fragmentation is seen in *ATR*^{-/-} cultured blastocysts. Conversely, *ATM*^{-/-} mice are viable (Y. Xu et al. 1996). This suggests a fundamental role for ATR-dependent signaling in cellular homeostasis that cannot be complemented by ATM.

Mouse models have been generated in order to investigate the consequences of ATR depletion. *Atr* knockdown in the adult mouse, using recombinant Cre technology, revealed an age-related phenotype, and stem cell depletion in tissues with high replicative capacity, with little impact on brain tissue (Ruzankina et al. 2007). Age-related features such as hair greying, kyphosis and osteoporosis were also observed in this model.

In a different approach, a mouse model was developed incorporating the human ATR-Seckel syndrome splicing mutation (*ATR*^{S/S}), first described by O'Driscoll and colleagues (O'Driscoll et al. 2003). *ATR*^{S/S} mice were born at sub-mendelian ratios, and importantly, faithfully reproduced the clinical features manifested in the human syndrome. These include severe growth retardation and microcephaly with receding forehead and micrognathia. *ATR*^{S/S} mice also exhibit signs of premature ageing and have reduced longevity, compared to control littermates (Murga et al. 2009). Additionally, signs of embryonic replicative stress were observed in this model, suggested by increased activation of DDR processes, such as H2AX phosphorylation, 53BP1 foci formation and breakages in the chromosomes; and elevated apoptosis observed in *ATR*^{S/S} mouse embryonic tissues (Dummler et al. 2007; Murga et al. 2009). More recently, *Atr* deletion in the mouse nervous system revealed a selective impact on certain progenitor cells during neurogenesis, with particularly decreased cellularity in the brain cortex and *corpus callosum* (Lee et al. 2012). Increased DDR was also observed, and the effects were attributed to high replicative stress in these rapidly proliferating cell populations.

1.4.1. Key ATR substrates in the DDR

Activated ATR can phosphorylate a number of downstream targets to modulate checkpoint activation, DNA repair and apoptosis (Figure 1.5). These include H2AX, CHK1, p53, BRCA1, NBS1. Large-scale proteomics analysis has revealed a high number of novel substrates for ATR and/or ATM in response to DNA damage, vastly increasing the scope of pathways linked to the DDR (Matsuoka et al. 2007). Although the biological significance of these novel interactions is yet unknown, it is noteworthy that a number of these novel substrates are components of the insulin-mTOR network.

While the list of ATR and ATM substrates keeps increasing, I'll give here an overview of some of the key mediators of the ATR-dependent signaling in the DDR.

▪ H2AX:

Histone H2A variant X (H2AX) is a highly conserved variant of the core histone H2A. Phosphorylation of H2AX on Ser139 (γ H2AX) is an early step in the DDR, which can be mediated by ATM, DNA-PK and/or ATR in response to various types of DNA damage (Easton et al. 2005; Fernandez-Capetillo et al. 2003). An extended region of γ H2AX is then formed along the chromatin, surrounding the sites of DNA damage, which facilitates the association of the repair and checkpoint machineries (Rogakou et al. 1999; Unal et al. 2004). γ H2AX is generated in an ATR-dependent manner in response to damage caused by UV exposure or HU treatment, resulting in the formation of foci at replication forks during S phase (Ward and Chen 2001). γ H2AX foci also co-localize with many DDR proteins, such as BRCA1, 53BP1, MDC1, RAD51, NBS1 and the MRN complex (Fernandez-Capetillo, Celeste, and Nussenzweig 2003). γ H2AX is therefore important for maintenance of genome stability. This is further highlighted by the elevated chromosomal instability, DDR defects and growth defects observed in *H2AX*^{-/-} mice (Altomare and Testa 2005; Bassing et al. 2002; Celeste et al. 2003).

▪ **CHK1**

The checkpoint kinase CHK1 is an essential Ser/Thr kinase, related to CHK2. Both CHK1 and CHK2 are coordinators of the signaling pathways that mediate intra-S and G2/M checkpoint activation (see section 1.4.2 of the Introduction), but CHK2 function does not compensate for CHK1 loss (Hirao et al. 2002). This could be related to an essential role for CHK1 in fork stability during DNA replication, associated with ATR activation (Sørensen et al. 2003; Sørensen et al. 2005). CHK1 is activated by ATR (or ATM)-mediated phosphorylation on Ser317 and Ser345, both in unperturbed cell cycles as well as in response to DNA damage. CHK1 activation can also be reinforced by autophosphorylation (Clarke et al. 2005). Chromatin association and activation of CHK1 requires ATR-ATRIP dependent phosphorylation of the 9-1-1 complex, RAD17 and BRCA1 (Soliman 2005; Ball et al. 2005; Byun et al. 2005; J. Kobayashi 2004; Zou et al. 2002; You et al. 2002).

The mediator protein Claspin is a component of the replication machinery that interacts with RPA and RAD17 (Land et al. 2007; Lee et al. 2005; Wang et al. 2006). CHK1 binding to Claspin facilitates ATR-mediated CHK1 activation (Hudson et al. 2002; Kumagai et al. 2000). The Claspin-mediated CHK1 activation is also promoted by interaction with Tipin (Timeless-interacting protein)/Timeless complexes and Tipin/Claspin complexes at RPA-coated ssDNA regions.

Activated CHK1 dissociates from the chromatin, and localizes to the cytoplasm and centrosomes (Mendoza et al. 2011; Niida et al. 2007). Notably, CHK1 retention at the centrosome reduces CDK1 activation and therefore modulates entry into mitosis (Manning et al. 2003; Krämer et al. 2004) (Section 1.5 of the Introduction).

Similar to ATR, *Chk1*^{-/-} mice are embryonic lethal (Manning et al. 2005; Q. Liu et al. 2000). Ser345 phosphorylation of CHK1 seems to have an indispensable function in cell metabolism, as its ablation in cultured cells proves lethal, as opposed to Ser317 mutant lines (Wilsker et al. 2008).

▪ **p53**

The tumor suppressor p53 is a transcription factor that modulates pathways leading to either growth arrest or apoptosis (Meek et al. 2004). p53-responsive genes are involved in activation of cell cycle checkpoints, DNA repair and apoptosis.

After DNA damage, ATR (or ATM) directly phosphorylates p53, which is also targeted by CHK1/CHK2, promoting its dissociation from MDM2, a ubiquitin ligase that normally binds p53 and targets it for nuclear export and proteasomal degradation. MDM2 is also a p53-responsive gene, thus operating on a negative feedback loop. Modulation of the MDM2/p53 loop, p53 biochemical activity and/or the cellular environment, determine the p53-response outcome. Cell cycle arrest-promoting p53 targets include p21, a cyclin-dependent kinase (CDK) inhibitor. This results in an arrest in G1, via suppression of cyclin E-associated CDK2 activity. Conversely, p53-mediated programmed cell death involves the BH3-only protein PUMA (p53 upregulated modulator of apoptosis), the BH domain proteins Bcl-2-associated protein X (BAX) and Bcl-2 antagonist/killer (BAK) (Reinhardt et al. 2012). This is a powerful tumor suppressor pathway that normally impedes deregulated proliferation of damaged/transformed cells.

▪ **53BP1**

p53-interacting protein 1 (53BP1) is a p53 activating mediator protein with roles during replication, and is rapidly recruited to sites of DNA damage (Hay et al. 2004; Harper et al. 2007). Association of 53BP1 with γ H2AX provides scaffolding to concentrate DDR proteins to sites of DNA damage (Sakasai et al. 2008). 53BP1 is phosphorylated by ATM and serves to amplify ATM signal at DSBs, via accumulation of the MRN complex (Noon et al. 2010). 53BP1 plays a role in initiation and maintenance of the G2/M checkpoint in response to IR (Shibata et al. 2010).

The function of 53BP1 in the ATR-dependent DDR is less understood. UV or HU-treatment induce 53BP1 phosphorylation by ATR (Magnuson et al. 2012; Jowsey et al. 2007). 53BP1 foci formation during replication arrest in S-phase depend on both ATR and CHK1 function (Ruvinsky et al. 2006; Sengupta et al.

2004). At the replication fork, 53BP1 is involved in recruitment of Bloom kinase (BLM), and interacts with RAD51 in a CHK1-dependent mode to affect HR (Miron et al. 2001; Tripathi et al. 2008). 53BP1 is also thought to amplify ATR signaling in UV-induced checkpoint activation (Laplante et al. 2009; Stiff et al. 2008).

▪ **BRCA1**

BRCA1 was originally identified as a susceptibility gene for familial breast and ovarian cancer (Futreal et al. 1994). Since then, an essential role for BRCA1 in development has been established, as *BRCA1* deficiency is embryonic lethal (Gowen et al. 1996). BRCA1 is a large protein with established functions in the DDR and tumor suppression, interacting with a host of DNA repair and checkpoint proteins, including CtIP and the MRN complex (involved in DNA end resection)(Yun et al. 2009a), BARD1 and TopBP1 (replication-associated damage response) (Greenberg et al. 2006), and recruitment of RAD51 to sites of DNA damage (Yun et al. 2009b). These interactions are mediated by C-terminal repeat domains, termed BRCT domains (Manke et al. 2003; Cantor et al. 2001). BRCA1 contains an SQ/TQ cluster, which is phosphorylated by ATR/ATM, and CHK1/CHK2 (Foray et al. 2003; Li et al. 2000; Lee et al. 2000). BRCA1 is required for the ATR/ATM –mediated phosphorylation of some of its targets in response to DNA damage, such as p53, CHK1, CHK2 and NBS1 (Foray et al. 2003; Yarden et al. 2002). Additionally, *BRCA1* expression is modulated by p53 (Arizti et al. 2000). p53 and BRCA1 appear to regulate transcription from an overlapping set of target genes in response to DNA damage. For example, BRCA1 facilitation of p53 phosphorylation promotes transcription of the CDK inhibitor, p21, which activates and maintains checkpoint arrest at the G2/M border (Fabbro et al. 2008; Siliciano et al. 1997). Cells deficient in *BRCA1* exhibit defects in G2/M activation after IR-induced DNA damage (Sabatini et al. 2006; Xu et al. 1999). Additionally, disruption of BRCA1-ATRIP interaction also leads to G2/M checkpoint defects (Venere et al. 2007).

1.4.2 ATR-dependent cell cycle checkpoints

Cell cycle checkpoints are surveillance mechanisms that convey transient arrests at discreet phases of the cell cycle that concede time for repair of damaged DNA (Sancar et al. 2004). Alternatively, the outcome of checkpoint activation may be programmed cell death if the damage is deemed too severe. These arrests are activated in response to cellular stress, and work to prevent genomic instability and accumulation of damaged DNA.

ATR signaling can activate multiple checkpoint arrests, namely during S-phase, but also at the G2/M transition, of which I will give a brief outline.

- **The intra S-phase checkpoint**

ATR mediates an S-phase arrest in response to replication stress. ssDNA regions are generated normally during replication, where low-level ATR activation is thought to also have a role (Sørensen et al. 2004; Shechter et al. 2004b) (section 1.4.3). However, prevention of normal replication fork progression can occur when a DNA polymerase encounters a block or DNA lesion, causing replication fork stalling. This must be overcome to allow completion of DNA replication. MCM helicase-mediated unwinding of the DNA at sites of replication fork stalling generates long stretches of ssDNA regions, which are recognized by RPA, which recruits ATR-ATRIP. This signal amplifies ATR-CHK1 activation and downstream signaling (Plas et al. 2009; Paulsen et al. 2007), which contribute to downregulation of DNA replication as well as replication fork stabilization. Activation of ATR and CHK1 mediates downstream phosphorylation of CDC25A, targeting it for degradation involving the SKP1/Cullin/F-box (SCF) complex (Rowinsky 2004; Busino et al. 2003). This prevents CDC25A dependent removal of the inhibitory phosphorylation on CDK2 (threonine 14 and tyrosine 15 residues). Inhibition of CDK2/Cyclin (A or E) complexes downregulates DNA replication by reducing the association of CDC45 with the pre-replication complexes (Guba et al. 2002; Méndez et al.

2000; Costanzo et al. 2000). Inhibition of DNA synthesis delays S-phase progression and allows stabilization of replication fork structures.

ATR also phosphorylates BLM in response to stalled replication forks, and this phosphorylation is required for recovery from HU-induced replication blocks (Sengupta et al. 2004).

▪ **The G2/M checkpoint**

The G2/M checkpoint orchestrates a cell cycle arrest that prevents the cells from entering mitosis when harboring DNA damage. Much like the intra-S checkpoint, G2/M arrest arises from the coordinated action of multiple pathways to regulate the activation/inactivation of the CDK1-Cyclin B1 complex, also termed the mitosis promoting factor (MPF) (Porter et al. 2003). During normal cell cycle progression, the expression of Cyclin B1 is highly regulated, and it peaks in G2 phase (Lindqvist et al. 2009). This restricts the formation of the MPF complex at other stages of the cell cycle. Phosphorylated Cyclin B1 concentrates at the centrosomes in late G2 (Jackman et al. 2003; Toyoshima et al. 1998). Activation of the MPF requires phosphorylation of CDK1 on Thr161. This is mediated by the CDK-activating kinase (CAK) when a threshold level of MPF is reached (Tassan et al. 1994) (Figure 1.5). The subsequent proteolysis of CyclinB drives mitotic exit and reentry into prophase (Kapuy et al. 2009).

The CDC25 dual-specificity phosphatases mediate removal of the inhibitory Thr14 and Tyr15 phosphorylations on CDK1, a step required for CDK1 activation (Mailand et al. 2002). Thr14 and Tyr15 phosphorylation of CDK1 is mediated by the Myt1 and Wee1 kinases, respectively. The Polo-like kinase 1 (PLK1) also phosphorylates Wee1 and Myt1, resulting in Wee1 degradation (Watanabe et al. 2004) and Myt1 inhibition (Nakajima et al. 2003). Aurora A kinase and co-factor Bora are also involved in PLK1 phosphorylation and activation (Seki et al. 2008; Lindqvist et al. 2009).

The CDC25 phosphatases are therefore key regulators of mitotic entry, where a fine balance between mitosis-inhibitory and activating signals determines the final outcome for the cell. There are three mammalian CDC25 homologues -

CDC25A, CDC25B and CDC25C – that have both unique and overlapping functions (Mailand et al. 2002). CDC25A activity is required to maintain the threshold of CDK1 activity necessary for promoting mitosis; and this role is further supported by CDC25B and CDC25C function. In turn CDK1 activation is also thought to mediate CDC25A stabilization, CDC25C activation, and CDC25B localization (Schmitt et al. 2006). Threshold-dependent activation of CDK1-Cyclin B1 therefore promotes a positive feedback activation loop. (Lindqvist, Rodríguez-Bravo, and Medema 2009). In CDC25A overexpressing cells CDK1 kinase activity is increased and the transition from G2 into mitosis is accelerated (Timofeev et al. 2010). Conversely, CDC25A is specifically degraded/inhibited after DNA damage, resulting in mitotic arrest (Schmitt et al. 2006; Boutros et al. 2006) and this can be mediated by ATR/CHK1-dependent signaling, depending on the nature of the damage.

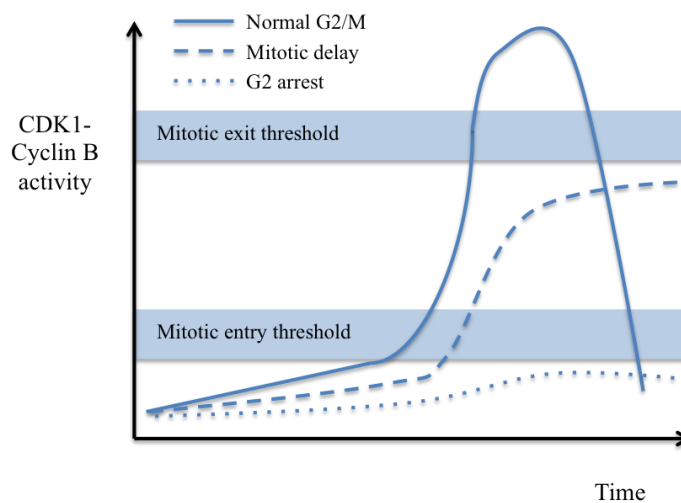


Figure 1.6 CDK1-CyclinB levels control entry into mitosis.

UV radiation and replication inhibitors can induce ATR-mediated G2/M arrest. Activated CHK1 phosphorylates CDC25A on Serines 76 and 123 (Donzelli et al. 2004; Zhao et al. 2002; Jin et al. 2008). CDC25A phosphorylation at these and other sites promotes its SCF-mediated ubiquitination and degradation (Busino et al. 2003). CHK1 can also phosphorylate CDC25C and promote its nuclear export (Peng et al. 1997); and Aurora A inhibition of CDC25B in response to DNA damage is also CHK1

dependent (Cazales et al. 2005). The result of all these actions is the sustained downregulation of CDK1/Cyclin B complex activation, which prevents entry into mitosis (Figure 1.6). ATR/CHK1 signaling therefore plays a key role in regulation of CDC25 phosphatase inhibition following certain types of DNA damage, resulting in mitotic arrest to allow time for repair.

Other checkpoints, including spindle assembly, have also been associated with ATR-dependent function (Smith et al. 2009).

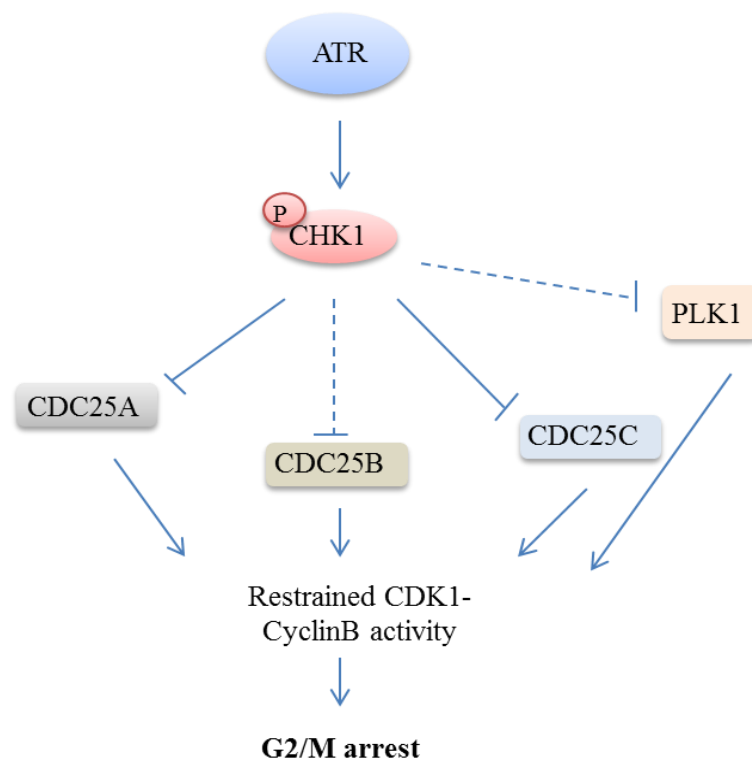


Figure 1.7 ATR-dependent G2/M checkpoint.

1.4.3 ATR signaling and replication

Both ATR/CHK1's absence is invariably lethal suggesting their essential involvement in unperturbed cell cycles (Cortez et al. 2001; Niida et al. 2007). A role for ATR in normal fork progression during DNA replication has been described, through modulation of origin firing. Single-stranded DNA is generated in normal replication forks during helicase progression and synthesis and joining of Okazaki fragments. ATR-ATRIP is attracted to RPA-bound ssDNA and activates CHK1, preventing late firing of origins of replication, by inhibiting the S-phase kinases CDK2 and CDC7. Thus, in every S-phase ATR is necessary to regulate the firing of origins of replication, stabilize replication forks and prevent early onset of mitosis (Shechter et al. 2004a; Paulsen and Cimprich 2007).

Accordingly, ATR-Seckel patient derived cells show reduced phosphorylation of H2AX and CHK1; reduced stabilization of replication forks after (aphidicolin-induced) replication fork stalling; and failure to undertake G2/M arrest (Alderton et al. 2004).

1.5 The centrosome

A number of ATR pathway components and other DDR proteins localize to the centrosome (Zhang et al. 2007).

The centrosome is a cellular organelle formed by the two centrioles and surrounding pericentriolar material (PCM), which is the main microtubule organization center in metazoan cells. The centrosome takes part in efficient cell cycle regulation (Hinchcliffe et al. 2001; Mikule et al. 2007; Matsumoto and Maller 2004), involved in processes such as spindle organization and orientation and accurate chromosome segregation during mitosis, although it is not essential (Doxsey et al. 2005). Centrosomal duplication is synchronized with initiation of DNA replication and each daughter cell should receive one functional centrosome after mitosis. Defects in centrosome maturation are associated with inability to form adequate bipolar spindles, or formation of monopolar spindles which can lead to polyploidy or failure to undergo cytokinesis (Doxsey et al. 2005; Shinmura et al. 2007; Uetake et al. 2004). Aberrant centrosomes have been linked to aneuploidy in cancer (Wang et al. 2004). Centrosomal function is thought to be essential during neurogenesis to maintain the proper balance between symmetric and asymmetric cell divisions, a determining factor in the regulation of brain size (Knoblich et al. 2010).

Additionally, the centrosome is involved in aspect of the cell cycle checkpoints. The CDK1-CyclinB complex is initially activated at the centrosome (Jackman et al. 2003), and the checkpoint kinase CHK1 has also been shown to accumulate at the centrosome in response to DNA damage and disruption of CHK1 centrosomal localization leads to G2/M checkpoint defects (Krämer et al. 2004; Löffler et al. 2007).

1.5.1 Pericentrin

The large conserved coiled-coil protein pericentrin (or kendrin) acts as an anchoring protein for a number of regulatory molecules and is an integral component of the pericentriolar material (PCM). Well described pericentrin interacting partners include the microtubule component γ -tubulin, the kinases PKA (Diviani et al. 2000), PKC β (J. Kim et al. 2008) and BCR-ABL (Patel et al. 2009) and the checkpoint kinase 1 Chk1 (Tibelius et al. 2009).

At the centrosome PCNT has been shown to have a role in the ATR-dependent checkpoint signaling (Zhang et al. 2007). Centrosome-associated CHK1 prevents/delays the transition from G2 to mitosis by inhibiting CDC25 activation of CyclinB-Cdk1 complexes, which accumulate at the centrosome during interphase (Delaval et al. 2010; Krämer et al. 2004). Current evidence suggests that pericentrin disruption would lead to loss of centrosomal CHK1, resulting in impaired G2/M checkpoint activation after DNA damage and premature entry into mitosis (Tibelius et al. 2009; Krämer et al. 2004). The subsequent accumulation of damaged DNA could eventually lead to mitotic arrest and cell death (Delaval et al. 2010).

This could lead to a reduction of total cellularity which would explain the severe dwarfism and microcephaly observed in humans and mice harboring *PCNT* mutations, supporting a role for impaired checkpoint signalling in primordial dwarfism (Microcephalic Osteodysplastic Primordial Dwarfism Type-II; MOPDII). Additionally, disruption of microtubule nucleation/organization function due to loss of γ -tubulin from the spindle poles can also result in spindle dysfunction during mitosis (Zimmerman et al. 2004). Aberrant spindle orientation could result in an imbalance between symmetric vs assymetric divisions and thus impair the balance of progenitor cells/differentiating cells during embryogenesis. Not only the deregulation of assymetric divisions in the neuroepithelion (Wang et al. 2009) could contribute to the observed microcephaly, but the reduced total cellularity could also explain the short stature observed in *PCNT*-mutated primordial dwarfism.

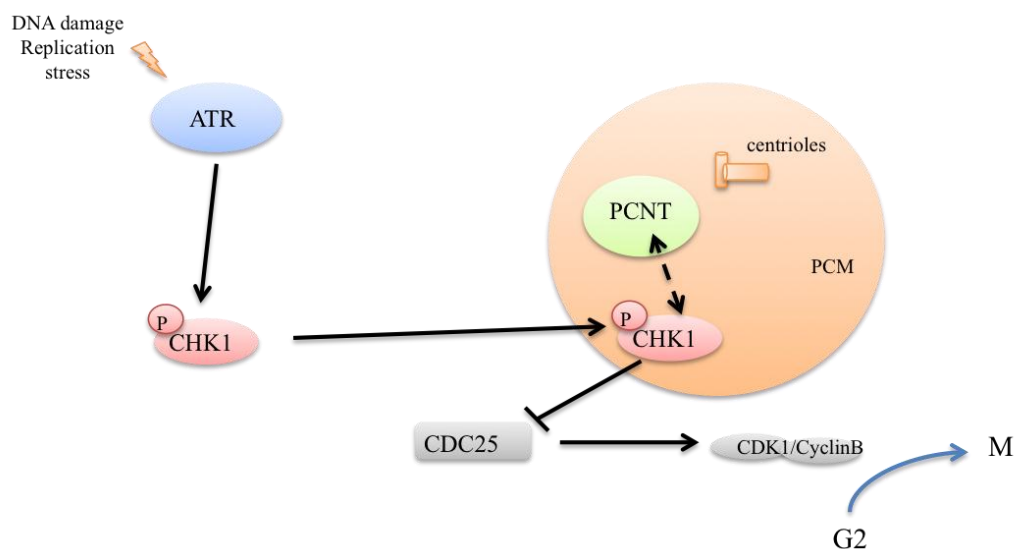


Figure 1.8 Model of pericentrin's role in the ATR-dependent G2/M checkpoint.

(adapted from Griffith 2008)

1.6 Cellular pathways that regulate growth

The development and growth of organisms requires the intricately coordinated action of a number of signaling pathways with impact on the size and patterning of cells, organs and organism. Established examples include the insulin/mTOR pathway and the MAPK pathway, of which I will here provide a concise overview. Other important growth-regulating signaling cascades involve the morphogens Wnt (Wingless) and TGF- β (Transforming growth factor) superfamily.

1.6.1 mTOR pathway and its role in growth/development

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase-related-kinase family (PIKK). mTOR is a key component of a signaling pathway that integrates inputs from nutrients and growth factors to regulate translation, cell growth and cell proliferation (Hay et al. 2004; Wullschleger et al. 2006; Martin et al. 2005).

Multiple extracellular signals impact on mTOR activation, such as insulin and growth factors (e.g. platelet derived growth factor (PDGF), insulin-like growth factors 1 and 2 (IGF-1/IGF-2))(Yang et al. 2008). Additionally, mTOR has also a role in sensing nutrient availability, energy levels and redox status in the cell (Yang et al. 2008; Land et al. 2007). When appropriate nutrients and growth stimuli are present, the mTOR pathway promotes cell growth by increasing ribosome biogenesis and protein synthesis. On the contrary, when nutrients are limiting, or in the presence of other kinds of stress, the cells suppress protein synthesis and promote nutrient recycling (autophagy) via mTOR signaling (Martin et al. 2005).

Extensive animal studies have supported the requirement of TOR function during development and growth. TOR deletion in *C. elegans* and *D. melanogaster* results in developmental arrest (Jia et al. 2004; Yang et al. 2007). In mammalian cells, growth to appropriate cell size requires mTOR and PI3K-dependent signals (Laplane et al. 2012). Additionally, homozygous *Tor*^{-/-} mouse embryos die shortly after implantation due to impaired cell proliferation (Murakami et al. 2004).

1.6.2 mTOR complexes 1 and 2

The mTOR protein kinase functions in most eukaryotic cells as the catalytic subunit of two distinct multi-protein complexes, named mTOR complexes 1 and 2 (mTORC1; mTORC2) (Kim et al. 2002; Laplante et al. 2009). The rapamycin-sensitive mTORC1 is composed of mTOR, the associated proteins RAPTOR (regulatory-associated protein of mTOR), mLST8 (also known as G β L), and also PRAS40 and DEPTOR (DEP domain-containing mTOR-interacting protein). mTORC2 also contains the catalytic subunit mTOR, mLST8/G β L, plus two different proteins - RICTOR (rapamycin-insensitive companion of mTOR) and mSin1 (also known as mitogen-activated-protein-kinase-associated protein1) (Figure 1.9).

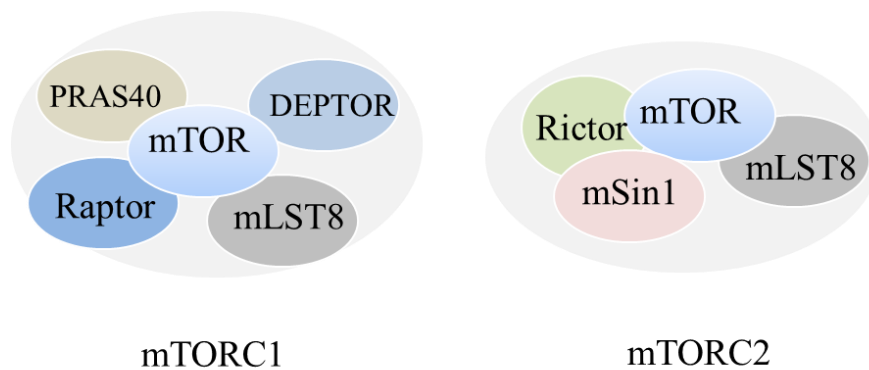


Figure 1.9 The mammalian TOR complexes, mTORC1 and mTORC2.

mTORC1 comprises the mTOR catalytic subunit in association with RAPTOR and mLST8 (also known as G β L), and the newly identified PRAS40 and DEPTOR. mTORC2 contains instead of RAPTOR a protein called RICTOR, but also other subunits such as mLST8 and mSin1.

mTORC2, which is for the most rapamycin insensitive, mediates signaling to downstream targets such as the protein kinase C α (PKC α), and is an important regulator of the cytoskeleton. mTORC2 is involved in modulation of cell proliferation and survival through an array of downstream substrates, including the transcription factors FOXO and MDM2, a regulator of p53 (Guertin et al. 2006). mTORC2 has also been shown to phosphorylate AKT on Ser473, facilitating AKT phosphorylation on Thr308 by the phosphoinositide dependent protein kinase-1 (PDK1).

Through its interaction with AKT, mTORC2 can also act as an upstream regulator of mTORC1 (Soliman 2005; Martin et al. 2005).

The mTORC1 complex has been more extensively researched. Its activity is stimulated by extracellular and intracellular signals that inform on the nutritional status of the cell or the organism. mTORC1 thus integrates different signals that include insulin and growth factors via outer membrane bound receptor tyrosine kinases (RTKs) , and amino acids and oxidative stress, via internal sensors such as the RAS homolog enriched in brain (Rheb) protein and the AMP-activated protein kinase (AMPK) (Martin et al. 2005).

Amino acid - induced mTORC1 activation involves the RAS-related GTPase (RAG) family of proteins. In the presence of amino acids, active RAG heterodimers interact with RAPTOR, and promote mTORC1 activation (Martin et al.2005).

mTORC1 mediates the insulin/IGF response via a signaling cascade involving the insulin receptor substrate (IRS), the phosphoinositide 3-kinase (PI3K), PDK1, AKT, the tuberous sclerosis complex (TSC1-TSC2) and Rheb (Figure 1.9). Rheb is a ubiquitously expressed small GTPase which in its GTP bound state directly binds to and activates mTORC1. However, nucleotide-free Rheb inhibits mTORC1, thus playing an essential role in mTORC1 signaling. Overexpression of Rheb can sustain mTORC1 activity in the absence of growth factor signaling (Martin et al. 2005; Kim et al. 2002; Shah et al. 2004).

mTORC1 can also be activated via the RAS-RAF-ERK1/2 pathway, downstream of receptor tyrosine kinases. Activated ERK1/2 (extracellular-signal-regulated kinases) inhibits the TSC1-TSC2 complex, the central negative regulator of mTORC1. Additionally, mTORC1 can integrate information of the levels of cellular energy (via AMPK) as well as hypoxia, by mechanisms also involving regulation of the TSC1-TSC2 complex activity, as described below (Land et al. 2007; Brugarolas et al. 2004).

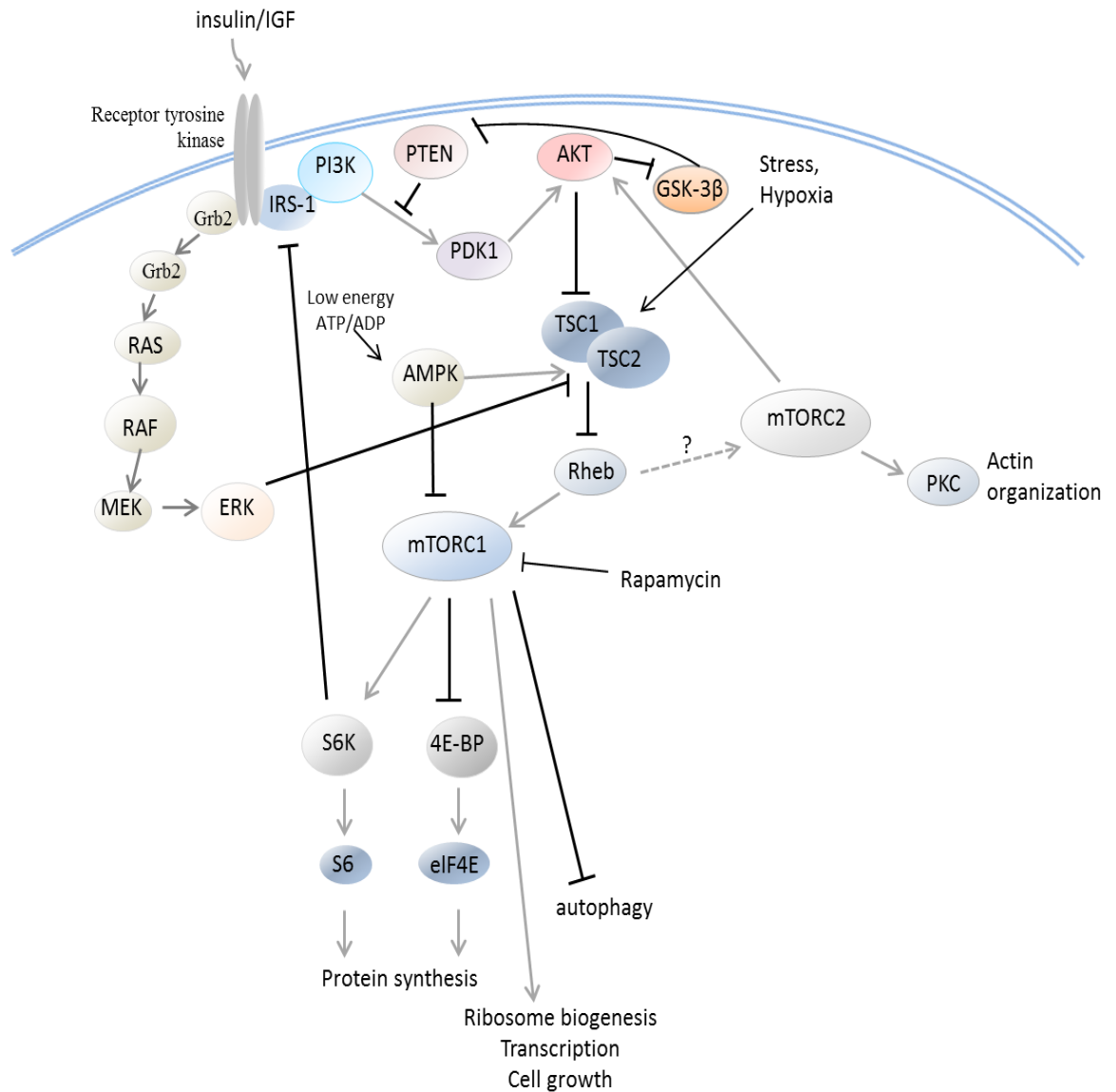


Figure 1.10 The insulin/mTOR signaling network involves negative feedback mechanisms and cross-talk with the PI3K/AKT and RAS/MAPK pathways.

Ligand binding induces phosphorylation of IRS-1 resulting in recruitment of the p110 catalytic subunit of PI3K. AKT phosphorylates and inhibits TSC1/TSC2 function, leading to mTOR activation. Through 4E-BP1, rapamycin-sensitive mTORC1 drives cell growth and proliferation by enhancing eIF4E-mediated mRNA translation. S6K1 activation by mTOR leads to IRS-1 phosphorylation and proteasomal degradation.

1.6.3 Key regulators of mTORC1

1.6.3.1 PI3K

The phosphatidylinositol 3-kinases (PI3K) are enzymes that can phosphorylate the 3'-inositol position on a number of membrane-associated phosphatidylinositols (PI). The canonical PI3K family is divided into three classes (I to III). The main subgroup activated by the receptor tyrosine kinases (RTKs) are the Class IA PI3K, whose heterodimers are composed of a catalytic subunit (p110 α , p110 β , or p110 δ), and a regulatory subunit (p85 α , p55 α , p50 α , p85 β , or p55 γ) (Emerling et al. 2011).

The lipid second messenger phosphatidylinositol 3,4,5 -triphosphate (PIP₃) is an important activator of signaling pathways that regulate growth and cell survival. PI3Ks are activated by G protein-coupled receptors (e.g. RAS) and RTKs such as the insulin receptor (Castellano et al. 2011). Upon ligand binding and activation of the receptor, activated PI3K can phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP₂) to generate PIP₃ at the plasma membrane. PIP₃ is an important signaling molecule. PIP₃ accumulation recruits the serine threonine kinases PDK1 and AKT to the plasma membrane enabling their activation. Besides AKT, PDK1 also phosphorylates the ribosomal protein S6 kinase (S6K) and the Serum/glucocorticoid regulated kinase 1 (SGK) (Rintelen et al. 2001; Mora et al. 2004). Germline deletion of *Pdk1* is embryonic lethal, whereas hypomorphic *Pdk1* mouse mutants are smaller in size and have a proportionately reduced organ volume (Lawlor et al. 2002). Interestingly, conditional *Pdk1* inactivation in the mouse brain causes microcephaly (Chalhoub et al. 2009).

1.6.3.2 PTEN

The phosphatase and tensin homolog (PTEN) acts in opposition of PI3K activity by dephosphorylating PIP₃ and therefore negatively regulating PIP₃-dependent signaling. PTEN functions as a tumor suppressor by inhibiting PI3K/AKT signaling, and therefore loss of PTEN results in increased PI3K signaling. *PTEN* deletion is embryonic lethal in mice (Di Cristofano et al. 1998). Mutations in *PTEN*

are associated with cancer predisposition, *macrocephaly*, and hamartomas in various tissues (Chalhoub et al. 2009), such as seen in Cowden's Disease.

Recently, PTEN's involvement in the DDR and DNA repair pathways has also been described (Ming and He 2012). PTEN has proposed roles in both double strand break repair and nucleotide excision repair, involving in this case suppression of XPC (Xeroderma pigmentosum, complementation group C) transcription (Ming et al. 2011).

PTEN can localize to the nucleus by several mechanisms (Planchon, Waite, and Eng 2008). There, it was shown to suppress Cyclin D activity (Chung et al. 2006); and interact with RAD51 and CENP-C, which would mediate PTEN's described involvement in DSB repair (Planchon et al. 2008).

Importantly, PTEN interacts with CHK1 (Martin et al. 2008); with CHK1 loss resulting in decreased PTEN phosphorylation and decreased total levels of PTEN. Conversely, PTEN depletion impairs CHK1-dependent checkpoint activation, resulting from cytoplasmic sequestration of CHK1 (Puc et al. 2005). PTEN interaction with p53 has also been observed. Under certain circumstances, PTEN regulates p53 protein levels, and transcriptional activity by modulation of p53's recruitment to the DNA (Freeman et al. 2003).

1.6.3.3 AKT

Upon receptor activation, AKT is recruited to the cell membrane through its affinity with PIP₃, where it is phosphorylated by mTORC2 at Ser473 and PDK1 at Thr308 (Xu et al. 2012). Once activated, AKT can phosphorylate a number of different substrates. Via its substrates BAD (Bcl-2-associated death promoter) and NF-κB ((nuclear factor kappa-light-chain-enhancer of activated B cells), AKT inhibits apoptosis and promotes cell survival (Datta et al. 1997; Madrid et al. 2000). AKT-dependent inhibitory phosphorylation of the glycogen synthase kinase (GSK-3) results in increased glycogen synthesis (Hajdуч et al. 2001). AKT also phosphorylates PRAS40, preventing it from inhibiting mTORC1 (Vander Haar et al. 2007).

AKT exists in 3 different isoforms (AKT 1-3). AKT1 and 2 have similar patterns of tissue-specific expression, with *AKT2* more highly expressed in insulin-target tissue like fat and muscle (Dummler et al. 2007). *Akt2*^{-/-} mice exhibit a severe

diabetic phenotype (Cho et al. 2001). *AKT3* expression is significantly lower than that of the other isoforms, with the exception of the brain and testes. *Akt3* knockout mice exhibit a significant reduction in brain size (Easton et al. 2005). Interestingly, mTOR signaling appears attenuated in the brains of *Akt3*^{-/-} but not *Akt1*^{-/-} mice (Easton et al. 2005). This suggests that AKT isoform-specific mTOR regulation may contribute differently to cell growth regulation in different tissues. Mouse knockout models suggest both specific as well as redundant roles for the AKT isoforms in growth and metabolism, but a single functional *Akt1* allele appears to be sufficient for viability in mice (Dummler et al. 2006).

AKT has also been specifically associated with endochondral ossification (Rokutanda et al. 2009). *AKT1* seems to have the biggest impact on skeletal development.

AKT activation is a commonly found genetic defect in human cancers such as ovarian (40-70%), multiple myeloma (90%), acute myeloid leukemia (Acute myeloid leukemia (70%) and thyroid (80–100%) cancer, associated with increased tumour cell proliferation, survival and invasiveness (Altomare et al. 2005; Vivanco et al. 2002). AKT activation was also found in 100% of the cases of anaplastic large-cell lymphoma investigated (Altomare et al. 2005).

1.6.3.4 The TSC complex

The TSC1-TSC2 tumour suppressors are the central negative regulators of mTORC1 activation, receiving signals from a number of kinases including AMP-activated kinase (AMPK), AKT, RSK1 and ERK and thus integrating the mTOR response with a complex network of other signaling pathways (Yang et al. 2007; Soliman et al. 2005). TSC1 (or hamartin) acts as a stabilizer of TSC2 (tuberin).

The TSC1-TSC2 complex acts downstream of PI3K and AKT signaling. Active AKT phosphorylates two residues on TSC2 (Ser 939 and Thr1462) thereby inhibiting TSC1-TSC2. This results in release of the TSC1-TSC2 inhibitory effect over mTORC1, enabling activation of mTORC1 downstream signaling.

AMPK is a sensor for cellular energy, being activated by an increase in the AMP:ATP ratio. Active AMPK phosphorylates TSC2 (and RAPTOR) resulting in mTORC1 inhibition (Soliman et al. 2005).

The TSC1-TSC2 complex mediates mTOR oxygen-sensing function, by a mechanism involving the HIF-1 α -regulated expression of REDD1, which activates TSC1-TSC2 (Land and Tee 2007). The mTOR pathway is a positive regulator of HIF-1 α , with evidence for regulation at the level of transcription, translation and protein stability (Hudson et al. 2002).

The TSC1-TSC2 complex also responds to ERK signaling, via RSK, which directly phosphorylates and inhibits TSC1-TSC2 (Mendoza et al. 2011).

The GAP (GTPase activating protein) domain in TSC2 stimulates the GTPase activity of Rheb. Activated TSC1-TSC2 increases the conversion of Rheb-GTP to Rheb-GDP. In its GTP-bound form, Rheb acts as an activator of mTORC1 (Manning and Cantley 2003).

TSC1, *TSC2* mutations cause the severe disorder tuberous sclerosis, characterized by the development of benign tumors (hamartomas) in many tissues.

TSC-deficiency causes increase in cell growth and proliferation due to lack of TSC-dependent mTORC1 inhibition. This desensitizes the cells from extracellular signals, such as growth factor signaling, thus encouraging cell proliferation even when conditions are unfavorable. However, feedback inhibition from mTORC1 to AKT has been shown to limit cell growth in TSC-defective tumors (Manning et al. 2005). Overexpression of *Tsc1* and *Tsc2* results in reduced cell and organ size (Potter et al. 2001). This effect was shown to be counteracted by *S6k* overexpression in *Drosophila* (Huang et al. 2008; Radimerski et al. 2002).

1.6.4 Downstream targets of mTORC1

The mTORC1 pathway regulates cell growth and size through downstream effectors by phosphorylating substrates such as the regulators of translation 4EBP1 (eukaryotic initiation factor 4E (eIF4E)-binding protein 1) and the ribosomal p70 S6 kinases (S6K1 and S6K2) (Hay et al. 2004).

mTORC1 *inhibits* 4E-BP1 by phosphorylation at multiple sites. In its non-phosphorylated state, 4E-BP1 is bound to eIF4E. Upon phosphorylation by mTORC1, 4E-BP1 releases its inhibitory effect over eIF4E, allowing it to bind to 5'-capped mRNAs and promote translation initiation (Bhandari et al. 2001; Gingras et al. 2001).

The S6K serine/threonine protein kinases are able to phosphorylate the S6 ribosomal protein, a component of the 40S ribosome, stimulating initiation of protein synthesis. S6K1 is directly phosphorylated by mTORC1 on two residues, Thr389 and Ser371. Additionally, PDK1 has also been shown to directly phosphorylate S6K1 on Thr229. S6K1 activity seems to require coordinated phosphorylation at these 3 sites by mTORC1 and PDK1 (Magnuson et al. 2012).

Other identified S6K1 substrates implicate S6K1 activity in other processes, such as mRNA processing (via SKAR and CBP80), initiation of cap-dependent translation (via PDCD4, eIF4B and co-ordination with 4EBP1) and elongation (via eEF2 kinase); and nascent protein folding (via CCT β). In addition, S6K1 is linked to cell survival (via BAD, Mdm2, and GSK3) (Ruvinsky et al. 2006).

S6k1 null mice have reduced body and cell size, whereas the combined *S6k1* and *S6k2* knockout mice die perinatally. In *D. melanogaster*, *dS6K* inactivation causes severe developmental delay (pronounced body and organ size) and lethality (Miron et al. 2001).

Other important mTORC1 substrates include the transcription factor YY1 (Yin Yang1) and PGC-1 α (peroxisome-proliferator-activated receptor coactivator - 1 α) involved in expression of mitochondrial genes (Laplane and Sabatini 2009); the signal transducer and activator of transcription 3 (STAT3) (Zha et al. 2011); the hypoxia-inducible factor HIF-1 α , and SGK1 (serum and glucocorticoid-inducible kinase 1) which regulates progression through cell cycle via p27 (potentially regulated also via mTORC2) (Land et al. 2007; Hong et al. 2008).

1.6.4.1 mTOR feedback regulation loops

Feedback loops are important players in the regulation of mTOR signaling. Signaling from S6K1 is known to inhibit the insulin receptor substrate (IRS-1) by directly phosphorylating a number on inhibitory sites on IRS-1 (Wan et al. 2007; Briaud et al. 2005). IRS-1 phosphorylation on Serine 636 and Serine 639 promotes its proteasomal degradation, resulting in reduced signaling from the insulin/IGF receptor down to PI3K and its effectors. This negative feedback loop from S6K is known to down-regulate insulin signaling and may be involved in acquired insulin resistance in type II diabetes and obesity, as a result of sustained mTORC1 activation (Zick 2005). S6K1 signaling also represses *IRS-1* gene expression. S6K is also

involved in a positive feedback loop where it phosphorylates RICTOR at T1135, leading to AKT phosphorylation on Ser473 (Treins et al. 2010).

Additionally, mTORC1 can also phosphorylate the growth factor receptor-bound protein 10 (Grb10) resulting in feedback inhibition of PI3K signaling (Yu et al. 2011).

1.6.5 mTOR and human disease

Inappropriate mTOR pathway signaling is a known contributing factor to various human pathologies, notably cancer (Figure 1.11; Tables 1.3 and 1.4).

The mTOR pathway signaling is hyperactive in many cancers, particularly those associated with high PI3K signaling or with mutations in the *PTEN* tumor suppressor (Sabatini et al. 2006). Loss of PTEN function is associated with an array of cancer types including among others, thyroid, melanoma, ovarian, breast, brain and renal carcinomas. Amplified AKT and PI3K signaling is also a common feature of certain cancers (Table 1.3), and interestingly, BCR-ABL translocation and the amplification of genes encoding HER-2 or the epidermal growth factor receptor (EGFR) have also been shown to activate PI3K signaling (Guertin and Sabatini 2005; Dazert and Hall 2011).

Table 1.3 Oncogenes and tumor suppressor genes in the mTOR pathway.

(adapted from Dazert 2011)

Most frequent types of cancer	
<u>Oncogene</u>	
<i>EGFR</i>	Glioblastoma; lung cancer; peritoneal cancer
<i>HER2</i>	Breast cancer; Ovarian cancer; Gastric cancer
<i>PIK3CA</i>	Breast cancer; head and neck squamous cell carcinoma; endometrial cancer
<i>AKT</i>	Head and neck squamous cell carcinoma; pancreatic cancer; ovarian cancer
<i>RAS</i>	Pancreatic cancer; thyroid cancer; colorectal cancer
<i>RAF</i>	Melanoma; thyroid cancer; ovarian cancer
<u>Tumor suppressor</u>	
<i>PTEN</i>	Glioblastoma; endometrial cancer; melanoma

The use of the mTOR inhibitor rapamycin and its analogs (“rapalogs”) has been widely investigated as a therapeutic tool for the treatment/management of tumors as well as other mTOR associated diseases (Plas and Thomas 2009). Studies have suggested that rapamycin treatment may be particularly effective in cancers associated with *PTEN*-inactivation; and a very positive response to rapamycin has been seen in particular in mantle-cell lymphoma (associated with Cyclin-D1 overexpression) (Rowinsky et al. 2004).

Table 1.4 Tumor-prone syndromes associated with mTOR signaling.

(adapted from Guertin et al. 2005)

Disease	Genetic defect	Pathology
Tuberous sclerosis complex (TSC)	<i>TSC1/TSC2</i>	Multiple hamartomas
LAM (lymphangiomyomatosis)	<i>TSC2</i>	Abnormal lung cell proliferation
Cowden’s disease	<i>PTEN</i>	Hamartomatous tumor syndrome; macrocephaly
Proteus syndrome	<i>PTEN; AKT1</i>	Hamartomatous tumor syndrome; overgrowth
Lhermite-Duclos disease	<i>PTEN</i>	Hamartomatous tumor syndrome
Peutz-Jehgers syndrome	<i>STK11/LKB1</i>	gastrointestinal hamatoma tumor syndrome; (STK11 is an AMPK regulator, which modulates TSC2 function)
HCM (familial hyperthrophic cardiomyopathy)	<i>AMPK</i>	Myocardial hyperthrophy

Interestingly, rapamycin has also been shown to suppress angiogenesis (Guba et al. 2002). This has been linked to mTORC1’s role in the regulation of HIF-1 α . An hypoxic microenvironment is often found in tumors, resulting in increased levels of HIF-1 α which in turn promotes the expression of the vascular endothelial growth factor (VEGF), involved in blood vessel formation (Bernardi et al. 2006; Hudson et al. 2002). Consistently, rapamycin treatment has proven useful in the management of Kaposi’s sarcoma, which is characterized by elevated VEGF signaling and strong vascularization (Campistol et al. 2004; Stallone et al. 2005), as well as tumors associated with loss of the Von Hippel-Lindau (VHL) tumor suppressor, a negative regulator of HIF-1 α (Thomas et al. 2006; Guertin et al. 2007).

Defects in mTOR are also known to underlie major growth defects, organ hypertrophy, diabetes, obesity and ageing (Dann et al. 2007; Dazert et al. 2011). As such, mTOR is a potential crucial therapeutic target for certain disorders associated with inappropriate cell or tissue growth; as well as graft rejection problems during transplants (rapamycin is a potent immunosuppressor).

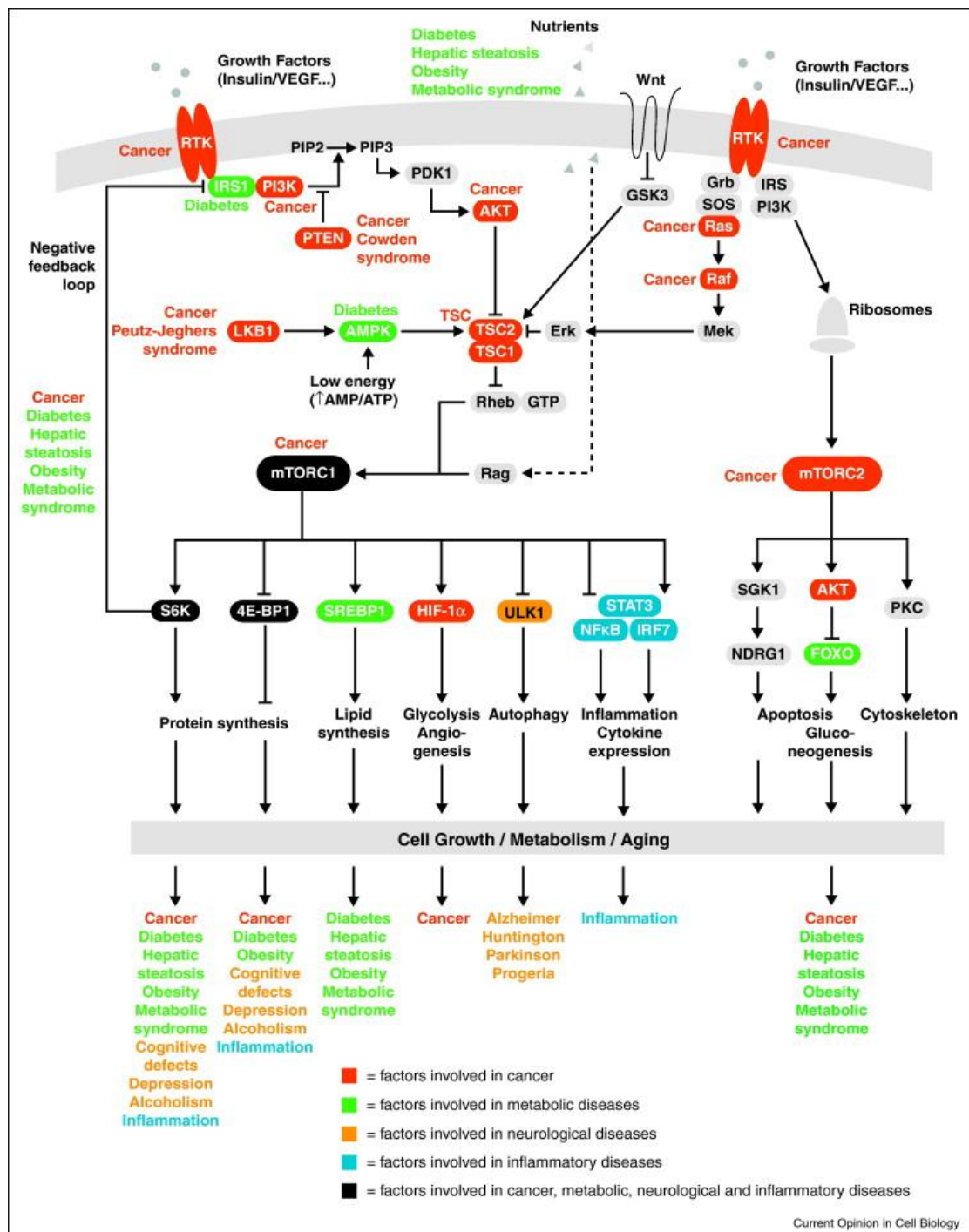


Figure 1.11 mTOR signaling in human disease.

(from Dazert 2011)

1.6.5.1 mTOR function in the brain

mTOR signaling has been implicated in several neurological disorders. These include Alzheimer's, Parkinson's and Huntington's disease, epilepsy and tuberous sclerosis, as well as a high number of brain tumors (Garelick and Kennedy 2011).

mTOR is expressed during early brain development, where it is involved in neuronal survival and differentiation, axon formation and synaptogenesis. The kinases PI3K and AKT are also important for neuronal migration and cortical lamination (Segarra et al. 2006). Conditional *PTEN* deletion in mouse neurons, associated with hyperactivation of mTOR pathway, results in macrocephaly, neuronal hypertrophy and other neuronal deficiencies (Kwon et al. 2001), and PTEN loss-of-function has also been observed in autism syndromes associated with macrocephaly (Butler et al. 2005).

TOR function in the adult CNS is important for synaptic plasticity, memory function, and control of food uptake (Garelick and Kennedy 2011; Tang et al. 2002). Increased mTOR activity in the hypothalamus suppresses food uptake and body weight (Cota et al. 2006), and chronic increased mTOR activity has been associated with metabolic disorders and homeostatic deregulation. In Chapter 7 I will present data on a human megalencephaly disorder caused by novel genetic defects in PI3K-AKT-mTOR pathway components.

1.6.5.2 Insulin/mTOR signaling and skeletal development.

Chronic hyperinsulinaemia results in mTORC1-dependent activation of S6K, which will inhibit insulin signaling at the cell membrane via the S6K-IRS feedback loop (Zick 2005; Ueno et al. 2005). As a result, PI3K and AKT are not properly activated leading to decreased glucose uptake. This mechanism is linked to the development of type II diabetes, and mice lacking *S6k1* exhibit enhanced insulin sensitivity (Um et al. 2006).

The role of insulin/IGF signaling in growth and development has been widely researched (Oldham and Hafen 2003; Tamemoto et al. 1994). *Inr* knockout in *Drosophila melanogaster*, for example, results in a significantly reduced body size (Oldham and Hafen 2003).

Notably, although the occurrence of diabetes is not clear for Seckel Syndrome patients, unpublished evidence suggests it. *Acanthosis nigricans*, a hyperpigmentation of the skin commonly caused by insulin resistance, has been observed in Seckel syndrome patients. The ATR-Seckel mouse also has a depressed insulin-dependent-transcriptional axis (Murga et al. 2009). ATM defects are also known to affect insulin-dependent signaling and A-T patients have *diabetes mellitus*, indicating insensitivity to insulin signaling (Bar et al. 1978; Morrell et al. 1986).

Interestingly, the mTOR/AKT pathway has been shown to regulate the processes of endochondral ossification and skeletal growth, where the insulin pathway has a pivotal role (Phornphutkul et al. 2006). Links between insulin/mTOR pathway signaling and skeletal development will be further discussed in Chapter 4.

1.6.6 RAS/MAPK pathway and its role in growth/development

The RAS/mitogen activated protein kinase (MAPK) pathway is an important regulator of cell growth and differentiation during normal development.

Activated RTK's function as docking sites for a number of intracellular proteins, including the SH2-domain containing SHC, SHP-2 and GRB2 (Lowenstein et al. 1992). The subsequent recruitment of SOS promotes activation of RAS (from inactive GDP-bound to active GTP-bound). RAS-GTP promotes signaling via the MAPK pathway, which includes the kinases RAF, MEK and ERK. ERK dependent phosphorylation of cytoplasmic as well as nuclear substrates (including cell cycle regulators) mediates cell proliferation (F. Chang et al. 2003). Some of ERK1/2 targets include the ribosomal protein S6 kinases (RSKs), mitogen- and stress-activated protein kinases (MSKs), and ternary complex factors (TCFs). Additionally, ERK signaling regulates the activation of mTORC1 via the phosphorylation and inactivation of TSC2 (Sunayama et al. 2010; Ma et al. 2005).

Germline mutations in RAS/MAPK pathway components have significant impact on development and are responsible for a class of developmental syndromes, aptly named 'RASopathies' (Table 1.5) usually associated with cardio-vascular abnormalities and neurocognitive delay, as well as growth problems (Tidyman et al.

2009; Tidyman et al. 2008; Cesarini et al. 2009). Most of these mutations result in increased Ras/MAPK signaling. Additionally, there is an increased risk of developing cancer. *RAS* somatic mutations alone have been observed in around 20% of all malignancies (Tidyman et al. 2009).

Table 1.5 Genetic syndromes of the RAS-MAPK pathway.

(adapted from Tidyman et al. 2009)

Syndrome	<i>RAS-MAPK pathway gene</i>
Noonan	<i>PTPN11; SOS1; RAF1; KRAS;</i>
LEOPARD	<i>PTPN1; RAF1</i>
Hereditary gingival fibromatosis 1	<i>SOS1</i>
Neurofibromatosis 1	<i>NF1</i>
Capillary malformation-arteriovenous malformation	<i>RASA1</i>
Costello	<i>HRAS</i>
Autoimmune lymphoproliferative	<i>NRAS</i>
Cardio-facio-cutaneous	<i>BRAF; MAP2K1; MAP2K2; KRAS</i>
Legius	<i>SPRED1</i>

Despite the substantial knowledge that already exists of signaling pathways that govern growth, how organ and organism size is determined remains in large part unknown, and could benefit from the study of human growth disorders.

1.7 Aims

In the work presented in this thesis I investigate the consequences of defective ATR-dependent function, in particular how defects in this pathway manifest in the specific set of clinical features observed in forms of microcephalic primordial dwarfism. For this purpose, specific cell culture differentiation assays were designed and implemented using standard model cell culture systems, but also employing de-differentiation of patient-derived fibroblasts. My novel findings help establish a previously unsuspected role for ATR function in differentiation processes, specifically its involvement in skeletogenesis via chondrogenesis.

Clinical features linked to ATR-signaling deficiency include microcephaly and severe growth retardation, and the insulin/mTOR pathway is a crucial regulator of growth and developmental processes. As such, and in view of the significant overlap existing between these seemingly unrelated pathways, I used cells derived from long recognized and novel human disorders to investigate the functional interplay between the ATR-dependent DDR and the mTOR pathway.

Chapter Two

Materials and methods

2.1 Chemicals and equipment

Unless otherwise stated, all chemicals were obtained from Fisher Scientific, Invitrogen or Sigma, and all enzymes from New England Biolabs or Roche. Cell culture media was obtained from Gibco Invitrogen Cell Culture and cell culture flasks, cryotubes and dishes from Nunc. Protein gels and western blots were carried out using the Biorad Mini-PROTEAN III gel system.

2.2. Mammalian cell culture

2.2.1 Cell culture conditions

All cell lines were grown in a humidified atmosphere at 37°C with 5% CO₂. Cells were passaged every 2-4 days to maintain a logarithmic growth phase.

Adherent cells were washed once with PBS and incubated in trypsin solution (250mg/ml in PBS) at 37°C until detached. Cells were then washed with fresh culture medium, pelleted by centrifugation at 2000g for 2 min and re-suspended in an appropriate volume of fresh culture medium.

Lymphoblastoid cell lines (LBLs) were cultured in RPMI 1640 (Dutch modification medium without L-glutamine) supplemented with 15% fetal calf serum (FCS), 2mM L-glutamine, 500U/ml penicillin, 50µg/ml streptomycin (referred to as complete RPMI1640 medium). Primary fibroblast cell lines were cultured in MEM supplemented with 15% fetal calf serum (FCS), 2mM L-glutamine, 500U/ml penicillin, 50µg/ml streptomycin (referred to as complete MEM medium). MG63 cells were cultured in MEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 500U/ml penicillin, 50µg/ml streptomycin. T98G glioblastoma cells were cultured in MEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 500U/ml penicillin, 50µg/ml streptomycin, 1% non-essential amino acids (NEEA), 1% sodium pyruvate.

For long-term storage, cells were pelleted and re-suspended in culture medium containing 10% DMSO. 2ml aliquots containing approximately 5×10^6 cells were transferred to cryotubes and cooled slowly to -80°C before being stored in

liquid nitrogen. Cell lines were thawed rapidly in a 37°C water bath and re-suspended in 20mls of complete medium.

2.2.2 Cell lines

Table 2.1 Mammalian cell lines used in this study.

Cell line	Source/reference
<i>Fibroblasts</i>	
1BR.3 (WT)	GDSC cell bank
FO2-98 (<i>ATR</i> ; <i>ATR-S</i>)	(O'Driscoll et al. 2003)
ASB (<i>PCNT</i> ^{E220X/-} ; <i>PCNT-S</i>)	(Griffith et al. 2008)
42552 (<i>PCNT</i> ^{C1990fs/-} ; <i>PCNT-S</i>)	(Griffith et al. 2008)
(<i>ORC1</i> mut; MGS)	(Bicknell, Walker, et al. 2011)
CL10-0031 (<i>IFT43</i> mut; Sensenbrenner Syndrome)	A kind gift from Prof. H Brunner (Nijmegen, The Netherlands); (Arts et al. 2011)
<i>Lymphoblastoid lines (LBLs)</i>	
AG87 (WT)	GDSC cell bank
DK0064 (<i>ATR</i> ^{A2102G} ; <i>ATR-S</i>)	(O'Driscoll et al. 2003)
CV1559 (<i>PCNT</i> ^{E220X/-} ; <i>PCNT-S</i>)	(Griffith et al. 2008)
GM10080 (PTEN p.Glu261Ter; CD)	Coriell Cell Repository (USA)
GM16378 (<i>INSR</i> mut; DS)	Coriell Cell Repository (USA)
CAO133 (DS)	A kind gift from Dr. R Semple (Cambridge, UK)
48608/P3.1 (<i>STAMBP</i> ^{I25A>G/532C>T} ; <i>MIC-CAP</i>)	Dr. Kym Boycott (Ottawa, Canada)
LR07-155a1/P1.2 (<i>STAMBP</i> ^{299T>A/1270C>T} ; <i>MIC-CAP</i>)	Dr. Kym Boycott (Ottawa, Canada)
P5.1 (<i>STAMBP</i> mut; <i>MIC-CAP</i>)	Dr. Kym Boycott (Ottawa, Canada)
LR04-078/P7.1 (<i>STAMBP</i> ^{I12C>T} ; <i>MIC-CAP</i>)	Dr. Kym Boycott (Ottawa, Canada)
LR08-018 (<i>AKT3</i> ^{1391C>T} ; <i>MCAP/MPPH</i>)	(Rivière et al. 2012)
LR00-016a1 (<i>PIK3R2</i> ^{1117G>A} ; <i>MPPH</i>)	(Rivière et al. 2012)
LR05-204 (<i>PIK3CA</i> ^{1359_1361} del; <i>MPPH</i>)	(Rivière et al. 2012)
LR09-006 (<i>PIK3CA</i> ^{2740G>A} ; <i>MCAP</i>)	(Rivière et al. 2012)
02-22 (1q21.1 Dup)	(Harvard et al. 2011)
09-103 (1q21.1Del)	(Harvard et al. 2011)
<i>Other</i>	
MG-63 (human osteosarcoma)	American Type Culture Collection (ATCC)
T98G (human glioblastoma)	GDSC cell bank
C2C12 (murine myoblasts)	A kind gift from Prof. S Morley (Sussex, UK)

2.3 Differentiation assays

2.3.1 Osteoinduction

MG-63 osteosarcoma cells were cultured in different reported *osteogenic mediums, containing*:

- a) 5, 10 or 20 μ M of piceatannol, added to proliferating cells for a period of up to 3 weeks. Adapted from (J.-K. Chang et al. 2006).
- b) 4 μ g/ml dexamethasone; 100 μ M ascorbate; 10 μ M β -glycerophosphate (Mineralizing medium, MM), added to both confluent and proliferating cells. Adapted from (J. R. J. Adams, Sander, and Byers 2006)

and assessed at various time points for the induction of osteogenic-specific markers.

2.3.2 Detection of mineralization

Osteocyte-derived mineral deposition was assessed by Alizarin Red S (ARS) incorporation, which stains calcium-rich deposits in cells in culture. ARS recovery by acetic acid extraction was followed by semiquantification of staining measured by colorimetric detection at 405nm. Detection and quantification of mineralization was performed as described in (Gregory et al. 2004).

2.3.3 Chondroinduction

Patient-derived hTERT immortalized fibroblasts were chondroinduced (differentiated) by seeding in micromass culture (2×10^5 cells/ml) onto 24 well plates coated with the chondrogenic proteoglycan *aggrecan* (A1960, Sigma-Aldrich). Plates were prepared using 20 μ g of aggrecan/well, dried slowly overnight at around 37°C. Adapted from (French et al. 2004).

Aggregate sizes were measured using light microscope images (40X magnification) using Adobe®Photoshop® (arbitrary units, lower cut-off point at the single cell size approximately).

2.3.4 Measurement of sulfated glycosaminoglycan (GAGs)

The synthesis of chondrocyte-specific sulfated GAGs was determined using the dimethylmethylene blue (DBM) assay. Chondroinduced fibroblasts, cultured on aggrecan-coated plates as described above, were analyzed for the excretion of sulfated GAGs at 24h and 72h post-induction. The culture media from each individual well was removed and cleared of cells by centrifugation. DMB solution was prepared as described in (Carroll 1987). Sulfated GAG quantities were determined by comparison with a calibration curve of chondroitin sulfate solutions (in full media) used as standard. Semiquantification of staining was measured by colorimetric detection at 525nm. Adapted from (Barbosa et al. 2003).

2.4 siRNA mediated knockdowns

Logarithmically growing cells were trypsinized and 5×10^5 cells seeded onto wells on 6-well plates/small 2cm dishes in 2ml of medium. The oligonucleotides used were ON-TARGET plus SMARTpool siRNA, from Dharmacon (Table 2.2). Cells were transfected using METAFECTENE® PRO (Cambio) transfection reagent according to manufacturer's instructions. Cells were analysed at 24h post-transfection with repeat transfections done at 24h or 48h when necessary. The control oligonucleotide was ON-TARGETplus siCONTROL Non-targeting siRNA (Dharmacon).

Table 2.2 siRNA oligos

Target	Cell line	Oligo ref/NCBI accession number
<i>ATR</i>	1BR.3	L-003202-00-0005; NM_001184
<i>PCNT</i>	1BR.3	L-012172-00-0005; NM_006031
<i>Pcnt</i>	C2C12	L-062315-01-0005; NM_008787
<i>INSR</i>	MG-63	L-003014-00-0005; NM_000208
<i>GSK-3β</i>	MG-63	L-003010-00-0010; NM_002093
<i>STAMBP</i>	T98G	L-012202-00-0005; NM_006463 /NM_201647/ NM_213622

2.5 *STAMBP* lentiviral complementation

STAMBP-deficient MIC-CAP LBLs were complemented using Precision LentiORF viral particles, carrying the pLOC lentiviral vector incorporating Human *STAMBP* (cat no. OHS5901-101004516; clone ID PLOHS_100005246) from ThermoScientific. The infection was performed according to the manufacturer's instructions, and infected cells selected using Blasticidin. Successfully infected LBL cultures were determined by *STAMBP* immunoblotting.

2.6 Protein extracts

Whole cell extracts (WCE)

Cell pellets were washed 2X in PBS and either stored at -20°C or directly lysed in 50-100µL of lysis buffer:

- a) **IP extracts:** cells were resuspended in 50-100µL IP buffer (50mM Tris.HCl pH7.5, 150mM NaCl, 2mM EDTA, 2mM EGTA, 50mM NaF, 25mM β -glycerolphosphate, 0.1mM sodium orthovanadate, 0.2% Triton-X100, 0.3% Igepal, protease inhibitor cocktail (Roche)) and incubated on ice for 1hr, prior to either clearing by centrifugation or sonication.
- b) **Urea extracts :** cells were lysed in urea buffer (9M urea, 50mM Tris-HCl pH7.5, 10mM β -mercaptoethanol) and directly sonicated.

Cell lysates were centrifuged for 5 min at 13000 rpm at 4°C. The supernatant was transferred to a clean, cold eppendorf tube. The protein concentration was then determined using Bradford Protein Assay reagent (Bio-Rad Laboratories) at a UV absorbance of 595nm. Extracts were either stored at -80°C or directly boiled in 2x SDS loading buffer (5% SDS, 10% Glycerol, 10% β -mercaptoethanol, 125mM Tris-HCL pH6.8 and 0.2% bromophenol blue) and loaded onto SDS-PAGE gels.

2.7 CDC5A stability assay

Cells were either untreated or irradiated with 10J/m²/s U and then incubated in complete medium with 100µg/ml cycloheximide (CHX) for up to 4hrs. Cells were pelleted and resuspended in WCE extraction buffer in the continued presence of CHX. 50µg WCEs were resolved by SDS-PAGE and western blotted with anti-CDC25A antibodies (Alderton *et al.*, 2006).

2.8 PAGE/Western-blotting

Whole cell extracts (50µg, unless otherwise stated) were denatured by heating at 90°C for 10 mins in SDS loading buffer (50mM Tris pH6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and separated alongside molecular weight markers (Precision Plus Protein Prestained Dual Colour Standards, BioRad) by SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis). 6% to 15% acrylamide gels, with a 4% stack, were resolved at 100-160V in running buffer (25mM Tris-HCl, 250mM Glycine, 0.1% (w/v) SDS) using Mini-PROTEAN 3 apparatus (Bio-Rad).

Gels were transferred onto PVDF membranes (Immun-Blot® PVDF membranes for protein blotting, Bio-Rad) using a Trans-blot SD Semi-Dry Transfer Cell (Bio-Rad) in semi-dry transfer buffer (20% methanol, 30mM Tris-HCl, 200mM Glycine 0.03% SDS) for 1-1.5 hrs at 20-25V. Membranes were blocked in either 5% BSA or 5% non-fat milk in Tris Buffered Saline with 0.1% Tween-20 (TBS-T) for a period of 1hr. Membranes were then probed with primary antibody in 5% BSA/TBS-T overnight at 4°C with shaking. Membranes were washed with TBS-T, 3 times for 10 mins, and probed with HRP-conjugated secondary antibodies (DABKO) for 1 hr, followed by three further TBS-T washes. Labeled proteins were then detected by enhanced chemiluminescence (Pierce ECL Detection Reagents; or GE Healthcare ECL plus detection reagents).

2.9 Indirect Immunofluorescence

For indirect immunofluorescence (IF), LBLs were pelleted, swollen in 75nM KCl (10 mins) and fixed in 3% PFA, 2% sucrose. Following fixation, the cells were either stored in 70% EtOH at 4°C; or resuspended in PBS and immobilized onto poly-L-lysine coated slides by cytospinning. Adherent cells were cultured directly on cover slides and fixed in 3% PFA, 2% sucrose. Cells were permeabilized by addition of 150µl 0.1% Triton 100-X in PBS for 1-2 mins, washed in PBS by dipping 3X and blocked for 10 min in 150µl of 5% BSA/PBS. The cells were then incubated for 30 mins with 150µl of primary antibody in 5% BSA/PBS; washed in PBS by dipping, and a second blocking step performed for 10 min in 150µl of 5% BSA/PBS. 150µl of secondary antibody in 5% BSA/PBS were then added and the cells incubated for 30mins in the dark. For nuclear staining, 2ml of DAPI solution were added and incubated for 5 mins in the dark. The slides were again washed 3X in PBS and the excess liquid was removed. The slides were then preserved in anti-fading mounting media (Vectashield), and analysed using the Zeiss AxioPlan platform and images captured using SimplePCI software at constant exposure times.

2.10 Immunoprecipitation

In a final volume of 150µl, 500µg of WCE in IP extraction buffer (see 2.5) were immunoprecipitated with 1-2µg of the appropriate antibody. The mixture was agitated for at least 4hrs (to overnight) at 4°C. Protein G/Sepharose beads suspended in IP buffer were then for at least 1hr at 4°C. with constant agitation. After centrifugation at 4°C for 5 mins, the supernatant was discarded and the immunoprecipitate washed 3X with 300µl IP buffer. The immunoprecipitates were resuspended in 30-50 µl IP extraction buffer and 50 µl of 2X SDS loading buffer. The mixture was boiled for 10 mins to denature and release the bound protein. Immunoprecipitates were centrifuged at high speed and the supernatant resolved by SDS-PAGE and Western blotting.

2.11 Antibodies

Table 2.3 List of antibodies

Antibody	Company	Dilution (WB/IF)
ATR	Santa Cruz Biotechnology	1:300 WB
53BP1	Bethyl	1/200 IF
p-Rb S807/811	Cell Signaling	1:1000 WB
Rb	Cell Signaling	1:1000 WB
PCNT	Abcam	1:500 WB
HIF-1 α	BD Transduction Laboratories	1:500 WB
pAKT (Thr308)	Cell Signaling	1:1000 WB
pAKT (Ser473)	Cell Signaling	1:1000 WB
AKT	Cell Signaling	1:1000 WB
pS6K (Ser371)	Cell Signaling	1:500 WB
pGSK-3 β	Cell Signaling	1:1000 WB
GSK-3 β	Cell Signaling	1:1000 WB
p4E-BP1 (Thr37/46)	Cell Signaling	1:1000 WB
4E-BP1	Cell Signaling	1:1000 WB
pS6 (S240/S244)	Cell Signaling	1:1000 WB
pPDK1 (Ser241)	Cell Signaling	1:1000 WB
pPTEN (Ser380)	Cell Signaling	1:1000 WB
pTSC2 (Thr1462)	Cell Signaling	1:1000 WB
TSC2	Cell Signaling	1:1000 WB
pC-RAF (Ser259)	Cell Signaling	1:1000 WB
pPI3K p85 (Tyr458)/p55(Tyr199)	Cell Signaling	1:500 WB
PI3K p110 α	Cell Signaling	1:1000 WB
PI3K p110 β	Cell Signaling	1:1000 WB
PI3K p110 γ	Cell Signaling	1:1000 WB
PI3K p85	Cell Signaling	1:1000 WB
Insulin R β	Cell Signaling	1:500 WB
pCHK1 (S345)	Cell Signaling	1:500 WB
pCHK1 (Ser280)	Cell Signaling	1:500 WB
pCDK1 (Y15)	Cell Signaling	1:500 WB
CDC25A	NeoMarkers	1:300 WB
STAMBP	Santa Cruz Biotechnology	1:1000 WB
A-FK2 (Ub)	Enzo Lifesciences	1:100 IF
LC3A	Cell Signaling	1:500 WB / 1:100 IF
Caspase 3	Cell Signaling	1:500 WB
pC-RAF (S338)	Cell Signaling	1:1000 WB
pERK1/2 (T202/Y204)	Cell Signaling	1:1000 WB
ERK1/2	Cell Signaling	1:1000 WB
MEK1/2	Cell Signaling	1:1000 WB
AMPK- β 2	Cell Signaling	1:1000 WB
AMPK- β 1	Cell Signaling	1:1000 WB
pAMPK α (T172)	Cell Signaling	1:1000 WB
AMPK α	Cell Signaling	1:1000 WB
pACC (S79)	Cell Signaling	1:1000 WB
ACC	Cell Signaling	1:1000 WB
pRAPTOR (S792)	Cell Signaling	1:1000 WB
PIP3	MBL	1:100 IF
AKT3 (62A8)	Abcam	1:500 WB
PCNA	Santa Cruz Biotechnology	1:500 WB
DNA Polymerase α (POL1A)	Santa Cruz Biotechnology	1:200 WB
MCM2	Santa Cruz Biotechnology	1:1500 WB
β -tubulin	Santa Cruz Biotechnology	1:2000 WB
Anti-(rabbit/mouse/goat) HRP conjugated secondary antibody	DAKO	1:2000

2.12 HU-induced 53BP1 foci formation

LBLs were untreated or treated with 5mM Hydroxyurea for 2hr. Cells were then washed once in PBS and slides prepared using a cytospin. Cells were fixed in 3% PFA, 2% Sucrose for ten minutes followed by extensive PBS washing. Slides were then stained using the standard immunofluorescence technique described above using an anti-53BP1 primary antibody. Cells with > 5 53BP1 foci were counted as positive and the percentage of positive cells was assessed for each cell line.

2.13 G2/M checkpoint assay

Exponentially growing LBLs were washed with PBS and irradiated with 7J/m^2 UV-C or 3gy IR. Cells were then cultured in the presence of Colcemid for a further four hours in complete medium before harvesting, or for 24hr in complete medium with the addition of $1.5\mu\text{M}$ Nocodazole (Sigma). Cells were pelleted, swollen in 75mM KCL for 10 minutes and then fixed in 3%PFA, 2% sucrose for 15 mins. Cell nuclei were stained with DAPI and the cells were transferred into poly-L-Lysine coated slides (by cytospinning) and mounted using an anti-fading solution (Vectashield). The mitotic index was then determined for each cell line by counting at least 500 cells. A decrease in the number of mitotic cells was taken as indicative of activation of the G2/M checkpoint.

UV irradiation was performed using a UV-C source ($0.5\text{J/m}^2/\text{s}$). γ irradiation was performed with a ^{137}Cs source at a dose rate of $\sim 2\text{Gy/min}$.

2.14 Annexin-V apoptosis assay

For annexin V-apoptosis assessment I used the Single Channel Annexin V Apoptosis Kit (Alexa Fluor-488 conjugated anti-Annexin V with Sytox Green) from Life Technologies LTD (Paisley, UK) according to manufacturer's instructions.

2.15 Autophagy flux analysis

WT and patient LBLs were treated with the autophagy inhibitor Bafilomycin A1 for 2h to monitor autophagy flux. The cells were then harvested, and the urea WCE resolved by Western blotting and analyzed for the presence of the full length (LC3-I) and cleaved (LC3-II) forms of the autophagosome marker LC3A. Calculation of the LC3-II/LC3-I ratio gives an indication of the autophagy flux in the cells, and between the LBL lines.

2.16 RAS activation assay

Active RAS was detected using the RAS Activation Assay Kit from Millipore, according to the manufacturer's instructions. Fresh lysates from exponentially growing or serum-starved LBLs (MIC-CAP) were washed and cleared as per instruction in the appropriated buffers, and incubated with RAF-1 RBD Agarose, for the RAS-Pull-Down. The agarose beads were pelleted, washed, and resuspended in 40µl SDS-PAGE buffer before boiling and separated by SDS-PAGE.

2.17 Mitochondrial function

Analysis of mitochondrial function and mitochondrial mass in WT and patient LBLs was performed using the MitoTracker® dyes (Invitrogen) according to the manufacturer's instructions. Different mitochondrion selective probes were used. MitoTracker Red, for analysis of mitochondrial membrane potential; MitoSox, for detection of superoxide formation; and MitoGreen, which indicates total mitochondrial mass in the cells. The cells were incubated with the appropriate dyes as indicated, and fluorescence intensity was determined by FACS analysis.

2.18 Semi-quantitative RT-PCR

Semi-quantitative RT-PCR (24-28 cycles) was performed using the ProtoScript® AMV LongAmp® Taq RT-PCR Kit (New England Biolabs) using the primer sets indicated below.

Table 2.4 List of primers for RT-PCR.

Name	Primer sequence	T _m (°C)
Osteocalcin	Forward: 5' – ATGAGAGCCCTCACACTCCTC – 3'	60
	Reverse: 5' – GCCGTAGAAGCGCCGATAGGC – 3'	
Collagen I	Forward 5' – CATCTCCCCTTCGTTTTTGA – 3'	53
	Reverse: 5' – CTGTGGAGGAGGGTTTCAGA – 3'	
SOX9	Forward: 5' – GAACGCACATCAAGACGGAG – 3'	58
	Reverse: 5' – TCTCGTTGATTTGCTGCTC – 3'	
VEGFA	Forward: 5' – GTCTTGGGTGCATTGGAGCC – 3'	55
	Reverse: 5' – CCTCGGCTTGTCACATDTGC – 3'	
ELP4	Forward: 5' – AAGAGGATCCTGCCAACATTT – 3'	60
	Reverse: 5' – AGGATTGGATCCATCAAATCC – 3'	

2.19 Agarose gel electrophoresis

Agarose gels were made up at the required percentage (w/v) depending on fragment size, by dissolving Ultrapure Electrophoresis Grade Agarose (Invitrogen) in 1XTAE buffer (0.4 Tris acetate, 1mM EDTA). The mixture was heated with frequent mixing until dissolved, and after cool down ethidium bromide was added to a final concentration of 0.3µg/ml. The samples were prepared in loading buffer and subjected to electrophoresis in 1XTAE buffer. Samples were run alongside a 1K DNA ladder (Invitrogen) as size marker, and DNA was visualized using a UV transilluminator.

2.20 Quantitative RT-PCR (qRT-PCR)

qRT-PCR analysis for expression of chondrogenic markers was carried out using the QuantiFast SYBR Green PCR Kit (Qiagen) and the QuantiTect Primers listed below (Table 2.5).

Table 6 List of primers for qRT-PCR.

Name	Primer reference (QuantiTect, Qiagen)
<i>COL1A1</i> (NM_000088)	Hs_COL1A1_1_SG (cat no. QT00037793)
<i>SOX9</i> (NM_000346)	Hs_SOX9_1_SG (cat no. QT00001498)
<i>VEGFA</i> (NM_001025366)	Hs_VEGFA_6_SG (cat no. QT01682072)
<i>GAPDH</i> (NM_002046)	Hs_GAPDH_1_SG (cat no. QT00079247)

Reactions containing 12.5µl SYBR Green PCR Master Mix, 2.5µl 10XPrimer assay mix, 5µl RNase-free water and 5µl template cDNA to a final volume of 25µl were prepared in duplicate. Cycling was carried out using the Stratagene Mx3005 QPCR System. Cycling conditions: reactions were heated to 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Reactions were then heated up to 95°C for a further 1 minute and incubated at 55°C for 30 seconds.

Relative expression levels were calculated using the Ct (threshold cycle) values for the various conditions. The fold upregulation/downregulation following chondroinduction was calculated using the formula: $2e^{-\Delta Ct}$, with $\Delta Ct = Ct(\text{COL1A1, VEGFA or SOX9}) - Ct(\text{GAPDH})$.

Chapter Three

*Results I: The impact of defective ATR function on
skeletogenesis*

3.1 Introduction

The main clinical features observed in Seckel syndrome (SS) are the profound dwarfism/growth retardation, and a disproportionate impact on skeletogenesis (osteogenesis and chondrogenesis). Some authors have proposed Seckel syndrome as a chondrodysplasia (Rodríguez et al. 1982). Impaired ATR signaling appears to be a feature of SS patient cell lines (Griffith et al. 2008), and mutations in ATR were the first genetic defect identified in Seckel Syndrome (O'Driscoll et al. 2003). The index case exhibited skeletal abnormalities, including thoracic kyphosis, narrow iliac blades, *coxa valga*, subluxation of the hips, micrognathia and multiple ivory epiphysis of the hands. Indeed overt skeletal abnormalities (kyphosis, osteoporosis, micrognathia) are a feature of a humanized mouse model of ATR-mutated Seckel Syndrome (Murga et al. 2009).

I wanted to investigate whether these are simply ‘cell autonomous’ effects of loss of ATR since ATR is essential for cell division and survival, or whether ATR-signaling is specifically required for skeletogenesis and growth.

A role for ATR (and ATM) in regulating a spectrum of cellular processes beyond the DNA damage response (DDR) has been suggested. Recently, two large-scale proteomic studies have identified hundreds of proteins phosphorylated by ATM or ATR in response to DNA damage (Matsuoka et al. 2007; Stokes et al. 2007), but the significance of these phosphorylations is yet unclear. Many of these substrates are involved in protein modification, transcriptional regulation, and developmental processes. In particular, the exploration of ATR’s role in these processes has been hampered by its absolute requirement for survival in mice (Brown and Baltimore 2000). Ruzankina *et al.* have recently shown that mosaic ATR deletion in adult mice results in depletion of a high percentage of proliferating cells and an age-related phenotype including skeletal degeneration (Ruzankina et al. 2007).

3.1.1 Skeletogenesis

The formation and development of the skeleton is the result of tightly regulated differentiation and interaction between its three main constituting cell types: chondrocytes (which form cartilage); osteoblasts (deposit bone extracellular matrix) and osteoclasts (bone resorption). Whereas chondrocytes and osteoblasts are of mesenchymal origin, osteoclasts derive from the monocyte-macrophage cell lineage (Goldring et al. 2006). The activity, proliferation and differentiation of bone cells is carefully coordinated during embryogenesis, as bone is formed, and later in adult life in the process of bone remodeling. Migration of cells to the site of future bone formation is followed by cellular condensations that result of epithelial-mesenchymal interactions and finally differentiation of chondrocytes and osteoblasts (Ducy and Karsenty 1998). Several bone morphogenetics proteins (BMPs) have a role in the formation and shaping of mesenchymal condensations (Karsenty et al. 2008). In mammals, bone development occurs by two distinct mechanisms. Intramembranous ossification is the result of direct condensation of mesenchymal cells and accounts for a small number of skeletal elements, such as the flat bones of the skull and medial clavicles. Endochondral ossification generates the majority of bones and utilizes an initial cartilage template, on which osteoblasts differentiate to form endochondral bone (Figure 3.1.1) (Karsenty et al. 2008; Provot and Schipani 2005).

Chondrogenesis is the process that results in the formation of the cartilage intermediate. During endochondral bone development, chondrocyte differentiation takes place in the epiphysial growth plate (GP) at the extremities of long bones (Provot and Schipani 2007). Deposition of cartilage specific extracellular matrix (ECM) accompanies chondrocyte unidirectional proliferation, followed by hypertrophy, calcification, and ultimately cell death. Round proliferative chondrocytes develop along the longitudinal axis of the bone in a columnar layer and express collagen type II (Karsenty et al. 2008). The cells then gradually stop dividing and increase in size while becoming surrounded by the ECM. Differentiation into postmitotic hypertrophic cells (expressing primarily collagen type X) is accompanied by mineralization of the surrounding matrix (Goldring et al. 2006).

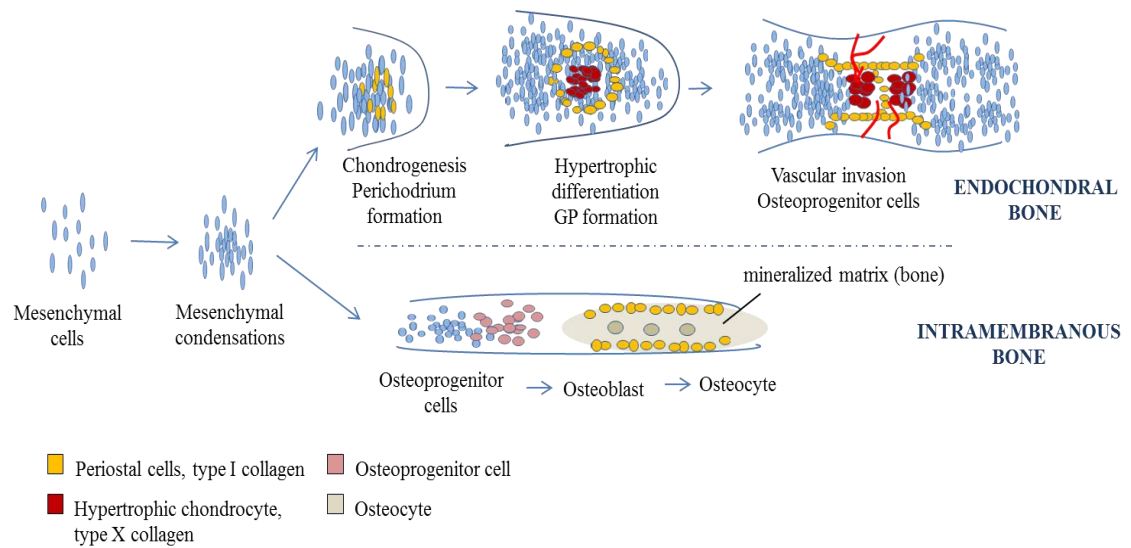


Figure 3.1.1 Mechanisms of bone formation.

Endochondral ossification requires the formation of a cartilage template, which is later replaced by bone, and is the mechanism by which the majority of the skeleton is formed. Bones of the skull are formed directly via intramembranous ossification without the use of a cartilage template.

Hypertrophic chondrocytes eventually die by apoptosis and are replaced by mineralized bone via vascular invasion, which allows recruitment of osteoclasts and osteoblasts, and consequent resorption of cartilaginous matrix and replacement with bone-specific matrix. Collagenous fibers and glycosaminoglycans (GAGs) such as chondroitin sulfate, hyaluronic acid (HA) and keratan sulfate, are the main organic components of the bone intercellular matrix, in addition to osteoblast proteins osteocalcin and osteopontin. 70% of the bone's dry weight consists of inorganic matter: Ca^{2+} phosphate (around 85%), CaCO_2 (10%), Ca and Mg fluorides (5%) (Provot and Schipani 2007; Provot and Schipani 2005; Zuscik et al. 2008; Adams, et al. 2007).

3.1.2. Regulation of chondrogenesis

The sequential stages of chondrogenesis and endochondral ossification involve a precise pattern of gene expression, under regulation of a number of transcription factors, as well as other signaling molecules such as growth and differentiation factors, as depicted in Figure 3.1.2. In the early stages, condensing mesenchyme already expresses ECM and cell adhesion molecules such as the IIA splice form of collagen II (COL2A), N-cadherin and tenascin C, as well as the important nuclear transcription factor SRY-box 9 (SOX9) (Karsenty et al. 2008). Non-hypertrophic proliferating chondrocytes then start expressing the IIB form of collagen II (COL2A2) and aggrecan, as well as collagen type XI and cartilage-derived retinoic-acid sensitive protein (CD-RAP), under SOX9 regulation (Karsenty et al. 2008). Aggrecan is the main ECM proteoglycan produced by chondrocytes, highly upregulated during chondrocyte differentiation and maturation. It interacts with hyaluronan filaments to form aggregates involving the cell-surface receptor CD44. An aggrecan core mutation has been identified in the homozygous nanomelic chick, resulting in extreme micromelia (i.e. disproportionately short limbs) with reduced head and trunk size and other major skeletal abnormalities (Schwartz et al. 1993).

SOX9 positively regulates proliferation and differentiation of non-hypertrophic chondrocytes, and negatively regulates hypertrophy, being excluded from mature chondrocytes. SOX5 and SOX6 can bind to and increase SOX9 transactivation function. *SOX9* haploinsufficiency causes Campomelic Dysplasia, a rare human disorder of severely impaired skeletal development (Foster et al. 1996). Upon withdrawal from the cell cycle, hypertrophic chondrocytes stop expressing collagen type II and instead express collagen type X and alkaline phosphatase (Karsenty et al. 2008). The runt-domain transcription factor, Runx2 is the main osteoblast differentiation factor, expressed at high levels in cells of the osteoblast lineage and in the adjacent perichondrium, where it regulates the expression of many genes that determine the osteoblast phenotype, including osteocalcin, an osteoblast-derived hormone expressed only in fully differentiated osteoblasts (Komori et al. 2008). Runx2 deficient mice show complete absence of bone formation and *in vitro* Runx2 gene expression promotes an osteoblast-specific gene pattern expression. Runx2 is also transiently expressed in prehypertrophic chondrocytes and has been shown to induce the expression of hypertrophic markers, like collagen type X, and is

required for mineralization of the cartilage matrix. Additionally, Runx2 is required for vascular invasion of cartilage, by means of activation of the vascular endothelial growth factor (VEGF) promoter (Karsenty et al. 2008; Komori et al. 2008; Lee et al. 2012).

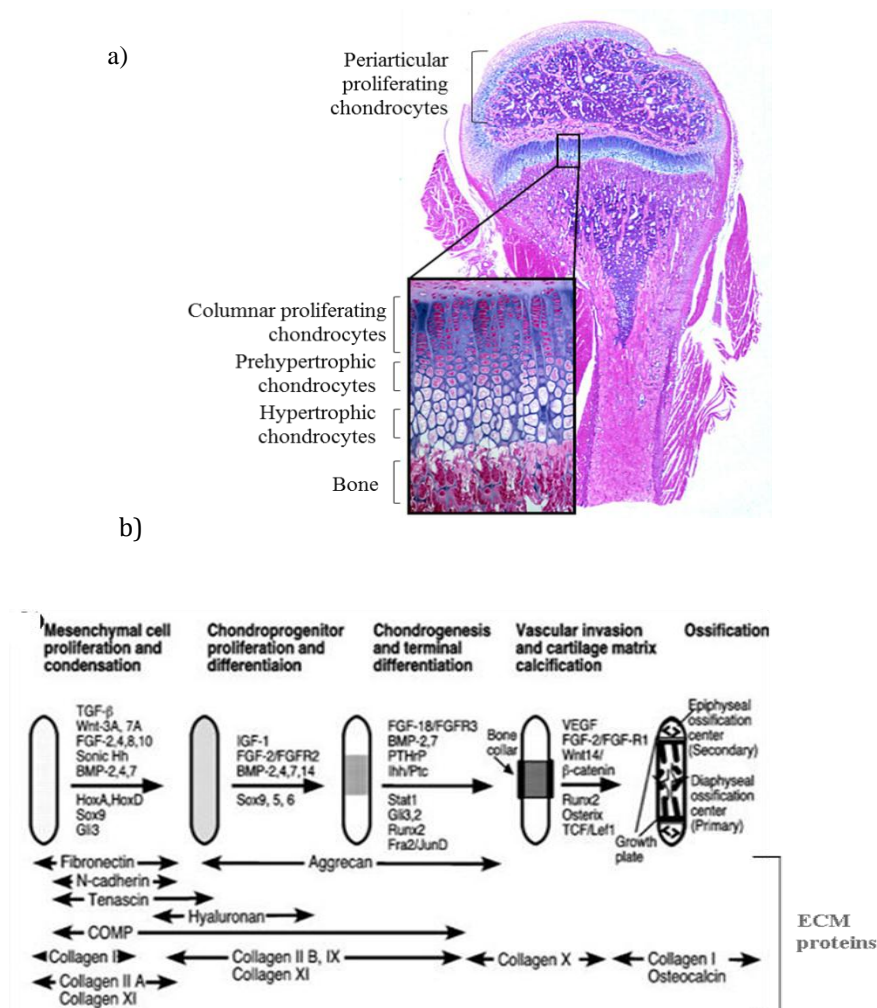


Figure 3.1.2 Endochondral bone formation.

a) Cellular organization of the fetal growth plate. **b)** Sequence of events of chondrogenesis during the development of long bones: different stages and accompanying patterns of growth and differentiation factors (above the arrows), transcription factors (below the arrows) and extracellular matrix proteins involved (adapted from Zuscik et al. 2008).

3.1.3. Hypoxia and VEGF expression in the fetal GP

The observation that the mammalian chondrocyte growth plate (GP) is highly hypoxic has led to the development of a new model of control of chondrocyte survival and differentiation, involving the hypoxia inducible factor, HIF-1 α (Provot and Schipani 2005; Schipani et al. 2001; Provot and Schipani 2007). HIF-1 is a heterodimer composed of HIF-1 α and HIF-1 β , the latter of which is constitutively expressed whilst the former is stabilized under low oxygen conditions.

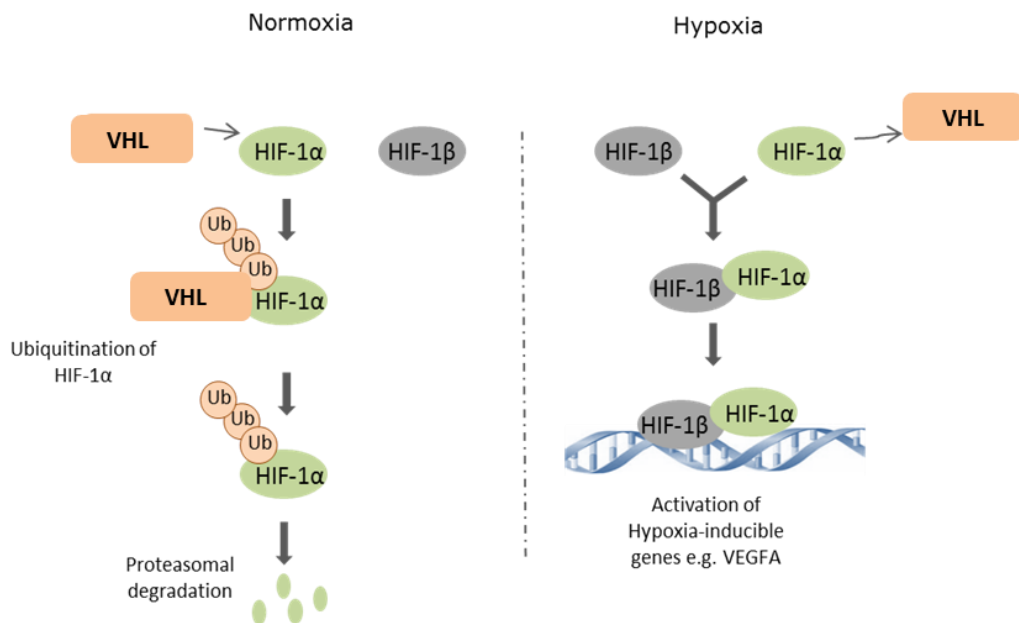


Figure 3.1.3 HIF-1 regulation under normoxic versus hypoxic conditions.

The hypoxia-inducible factor, HIF-1 operates as a transcription switch during regulation of oxygen homeostasis. Under normoxia the von Hippel-Lindau tumor suppressor protein (VHL) targets HIF-1 α for rapid ubiquitination and degradation by the proteasome. Under hypoxic conditions however, HIF-1 α is free to form a heterodimer with HIF-1 β , and the HIF-1 complex then recognizes and binds to the promoter of genes that mediate the hypoxia response, such as the gene encoding VEGF required for angiogenesis.

Under normoxic conditions, the ubiquitin ligase von Hippel-Lindau (VHL) targets the highly unstable HIF-1 α subunit to proteasome degradation (Figure 3.1.3). On the other hand, under hypoxia HIF-1 α is free to translocate to the nucleus where it activates the transcription of specific genes, including the hypoxic responsive elements (HRE), which include genes involved in glucose metabolism, angiogenic factors such as VEGF, cell cycle proteins and apoptotic factors. HIF-1 α is fundamental for the survival of hypoxic chondrocytes (Schipani et al. 2001).

Significant cell death is observed in HIF-1 α -deficient chondrocytes in the hypoxic center of the cartilage anlage, prior to hypertrophic differentiation, partly due to HIF-1 α regulation of VEGF expression in this region (Figure 3.1.4) (Provot and Schipani 2005; Schipani et al. 2001; Provot and Schipani 2007; Schipani 2006).

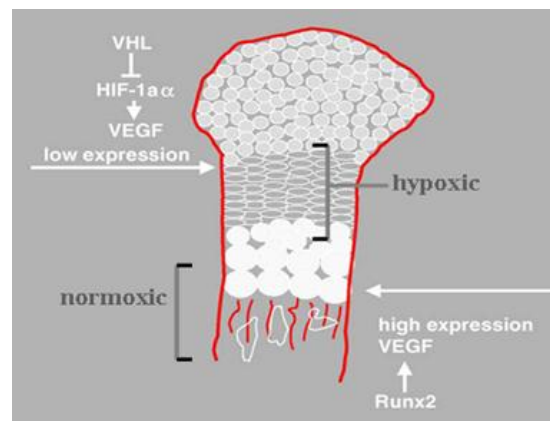


Figure 3 Hypoxia and VEGF expression in the fetal growth plate.

HIF-1 α controls VEGF expression in the hypoxic areas, whereas VEGF accumulation in the late hypertrophic chondrocytes is Runx2-regulated (adapted from Schipani 2006; Stiehl et al. 2002).

Other HIF-1 α targets are probably involved in the observed cell death of HIF-1 α -deficient growth plates, as VEGF-deficiency alone does not cause the same severe phenotype. HIF-1 α has also been shown to inhibit chondrocyte proliferation (Schipani et al. 2001) and stimulate ECM production with an increase in collagen type II accumulation. HIF-1 α dependent hypocellularity and decreased growth rate are the cause of dwarfism in mice with VHL deficient chondrocytes (Schipani 2006).

Other factors besides hypoxia may be involved in regulation of HIF-1 α function in chondrocytes, and indeed peptides such as insulin and interleukin-1 (IL-1) can also activate HIF-1 α under normoxic conditions, associated with PI3K/AKT pathway activation (Stiehl et al. 2002). In the context of chondrogenesis, a role for insulin and the insulin-like growth factor-I (IGF-I) has also been established, where at physiological concentrations they can induce chondrocyte differentiation *in vitro* (Phornphutkul et al. 2006).

A link to ATR-pathway function and HIF is provided by the fact that the ATR damage-response pathway has been recently shown to be activated by stalled

replication forks that result from hypoxia-induced S-phase arrest, involving phosphorylation of H2AX and accumulation of the tumour suppressor p53 (Hammond et al. 2002).

In this chapter I investigated the effect of impaired ATR function on osteogenesis and chondrogenesis using distinct model cell systems combining ATR-knockdown and patient-derived material in the attempt to understand the extreme skeletal phenotype observed in ATR-signaling deficient Seckel Syndrome patients.

3.2 Results

3.2.1 The effect of ATR deficiency on osteoinduction of MG63 cells

To assess the impact of ATR deficiency/impaired ATR function on osteogenic differentiation, we used an established cell system in osteoinduction studies, the human MG-63 osteosarcoma cell line, considered to be an undifferentiated osteogenic progenitor cell type. These cells are known to differentiate into mature osteoblasts in culture following treatment with “osteogenic” medium. The differentiation medium contains a source of phosphate, ascorbic acid, and dexamethasone which induces synthesis of osteoid in MG-63 cells (Adams et al. 2006)(see materials and methods). Recently, the polyphenol piceatannol was shown to induce differentiation in MG-63 cells associated with increase in bone morphogenetic protein 2 (BMP2), resulting in enhanced expression of alkaline phosphatase and osteocalcin (Chang et al. 2006).

Stable ATR knockdown was previously introduced in the MG-63 cells following transduction using the Expression ArrestTM pSM2 Retroviral shRNA mir system (Open Biosystems) (Figure 3.2.1a), and different strategies were tested to induce in these cells the expression of an osteoblast phenotype. This model line exhibited >90% knockdown of ATR protein whilst remaining viable.

Differentiation was monitored by analysis of expression of markers of osteoblast-like cells, such as increased expression of *osteocalcin*, *Runx2* and *collagen I*. MG-63 cells osteodifferentiation was first observed after prolonged (2 weeks) treatment with piceatannol, with the induction of expression of osteocalcin detected by semi-quantitative RT-PCR (Figure 3.2.1). A basal level of *osteocalcin* was detected in the control cells not unexpectedly since we are working with osteosarcoma cells. Surprisingly, the biggest increase in *osteocalcin* expression was detected in cells kept in confluence, in normal medium (MEM 10% FCS), and at more reduced time points, just after 24-48h after reaching confluence.

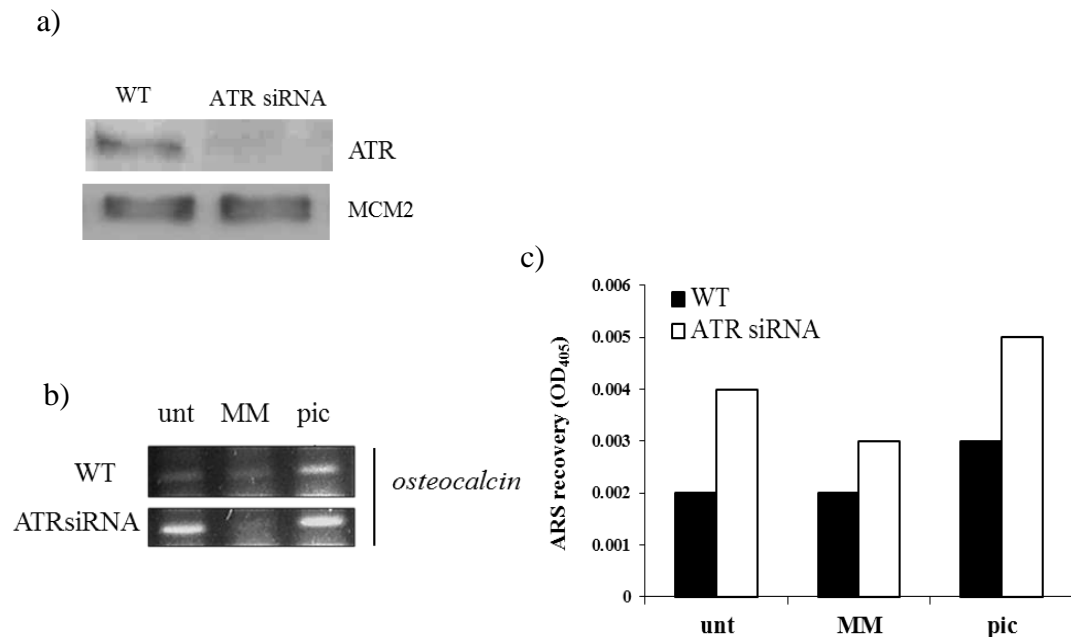


Figure 3.2.1. Different strategies tested to induce osteodifferentiation in MG63 cells.

MG63 control (WT) and ATR knockdown (ATR siRNA) cells were cultured in two different osteogenic supplemented media; 20 μ M of piceatannol (pic) alone or dexamethasone/ascorbate/ β -glycerophosphate (mineralizing medium, MM), and split every 2-3 days for a period of 2 weeks. **a)** Western blot analysis confirming ATR knockdown (>90%) in MG63 cells following siRNA. MCM2 levels were used for protein loading control. **b)** Expression of bone cell specific marker *osteocalcin* was assessed by semi-quantitative RT-PCR. Unexpectedly, exponential growing ATR siRNA cells appeared to express significant levels of *osteocalcin* compared to WT suggestive of premature engagement of osteogenesis here. Furthermore, upon piceatannol (pic) treatment levels appeared higher than those induced in WT. MM supplement was found to be a very poor inducer of *osteocalcin* expression. **c)** Mineralization was assayed by ARS quantification for the different culture conditions. I observed low levels of mineralization under these conditions, for both WT or ATR siRNA cells, with the ATR siRNA cells producing slightly more mineral than the WT.

The development of an osteoblast-like phenotype under these conditions was further confirmed by increased expression of a second marker of bone differentiation, collagen I (Figure 3.2.2). The pattern of expression of both osteogenic markers was identical between WT and ATR siRNA cells under these conditions.

No expression of either marker was detected after treatment with the mineralizing medium (MM), and their increase after piceatannol induction was

moderate when compared to the effect of simply growing the cells to confluence. The induction was sustained and detected up to 3 weeks of keeping the cells in confluence (Figure 3.2.2), with no apparent impact of *ATR* knockdown upon the expression of bone markers under these conditions, suggesting that at least in the earlier stages of osteogenesis *ATR* inhibition does not compromise osteogenesis.

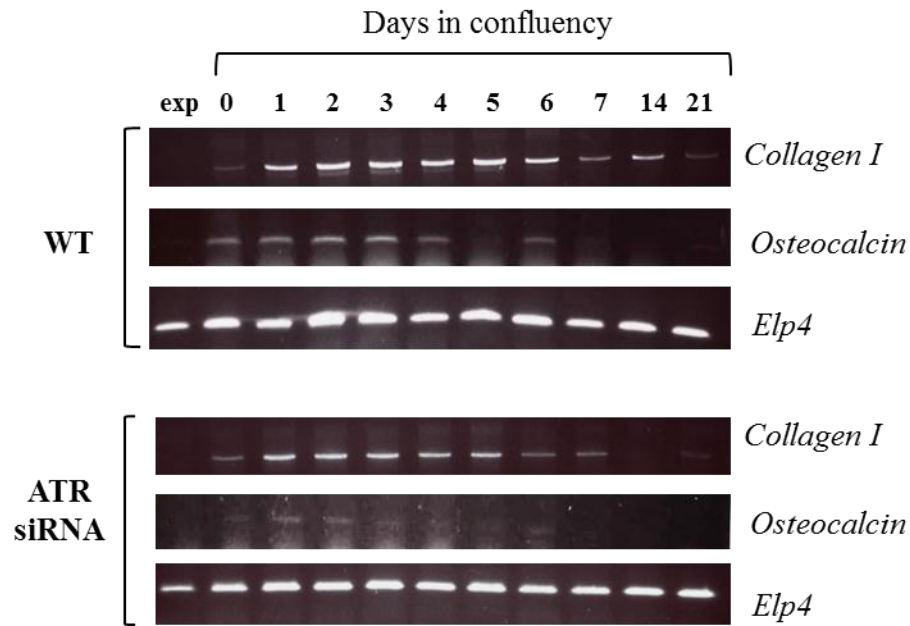


Figure 3.2.2 Kinetic expression of bone differentiation markers in confluent MG-63 WT and ATR siRNA cells.

Time dependent effect of confluence on osteogenesis was assessed in MG-63 WT and ATR siRNA. Confluent WT and ATR siRNA MG63 cells start expressing the osteogenic specific markers osteocalcin and collagen I after 24h in confluence. Total RNA was extracted after the appointed times and *osteocalcin* and *collagen I* expression was detected by semi-quantitative RT-PCR. The expression the housekeeping gene *Elp4*, component of RNA PolII holoenzyme, was used as an input control.

The later stages of bone formation require mineralization of osteoblasts, by accumulation of Ca^{2+} and inorganic phosphate, required for the formation of the bone main inorganic component, hydroxyapatite. Induction of mineralization has been observed in cultured cell lines after treatment with osteogenic medium containing a source of inorganic phosphate. In particular, MG-63 cells can be induced to mineralize both after piceatannol treatment (Chang et al. 2006) as well as in medium supplemented with a source of phosphate, ascorbic acid, and

dexamethasone (MM) (Adams et al. 2006). Mineral deposition can be assessed by a number of means including Von Kossa staining and Alizarin Red S (ARS) incorporation (Gregory et al. 2004). Both techniques were used to test mineralization in our system.

No mineral deposition was observed in von Kossa-stained monolayers of MG-63 cells grown both in the untreated as well as in the mineralizing medium with piceatannol, likely due to the short incubation times used (data not shown). However, Alizarin Red S staining revealed the formation of a mineralized layer in the cells cultured in osteogenic medium. At 2 weeks post-osteoiduction there was no detectable mineralization in exponentially growing cells, both untreated or stimulated with piceatannol/MM. However, the cultures kept in confluence showed an increase in ARS staining, indicating initialization of mineralization (Figure 3.2.3a). Moreover, the accumulation of mineral by the differentiating osteoblasts increased over time in culture as shown in Figure 3.2.3b. Quantitative analysis of ARS staining revealed that the cells treated with the osteogenic supplements produced more mineral than the untreated cells, particularly for the later time-points tested (Figure 3.2.3.c).

The level of ARS staining was overall significantly higher in the ATR siRNA MG-63 cells grown in mineralizing medium, with an accentuated decrease observed after 3 weeks. The mechanistic basis of this decrease is unclear but could imply a problem with maintenance of the osteogenic status following ATR siRNA in these cells. This could be consistent with the osteoporosis observed in various mouse models of ATR-deficiency. It must be taken in consideration that some cell death was observed in these cells after 3 weeks growth in confluent monolayer. By extending this study up to 8 weeks we observed that mineralization in the WT cells increases continuously over time. The ATR knockdown cells seemed to be producing more mineral initially, but at the later time points they fall below the WT cells significantly. Overall this data seem to indicate that initiation of osteogenesis is not defective after ATR inhibition, as both WT and ATR siRNA MG-63 cells are able to reach the final stages of osteoblast differentiation and mineralize. However, there is some modest evidence for a problem with maintenance of differentiation in the context of ATR knockdown, which would be consistent with the osteoporosis observed following ATR knockdown in adult mice (Ruzankina et al. 2007).

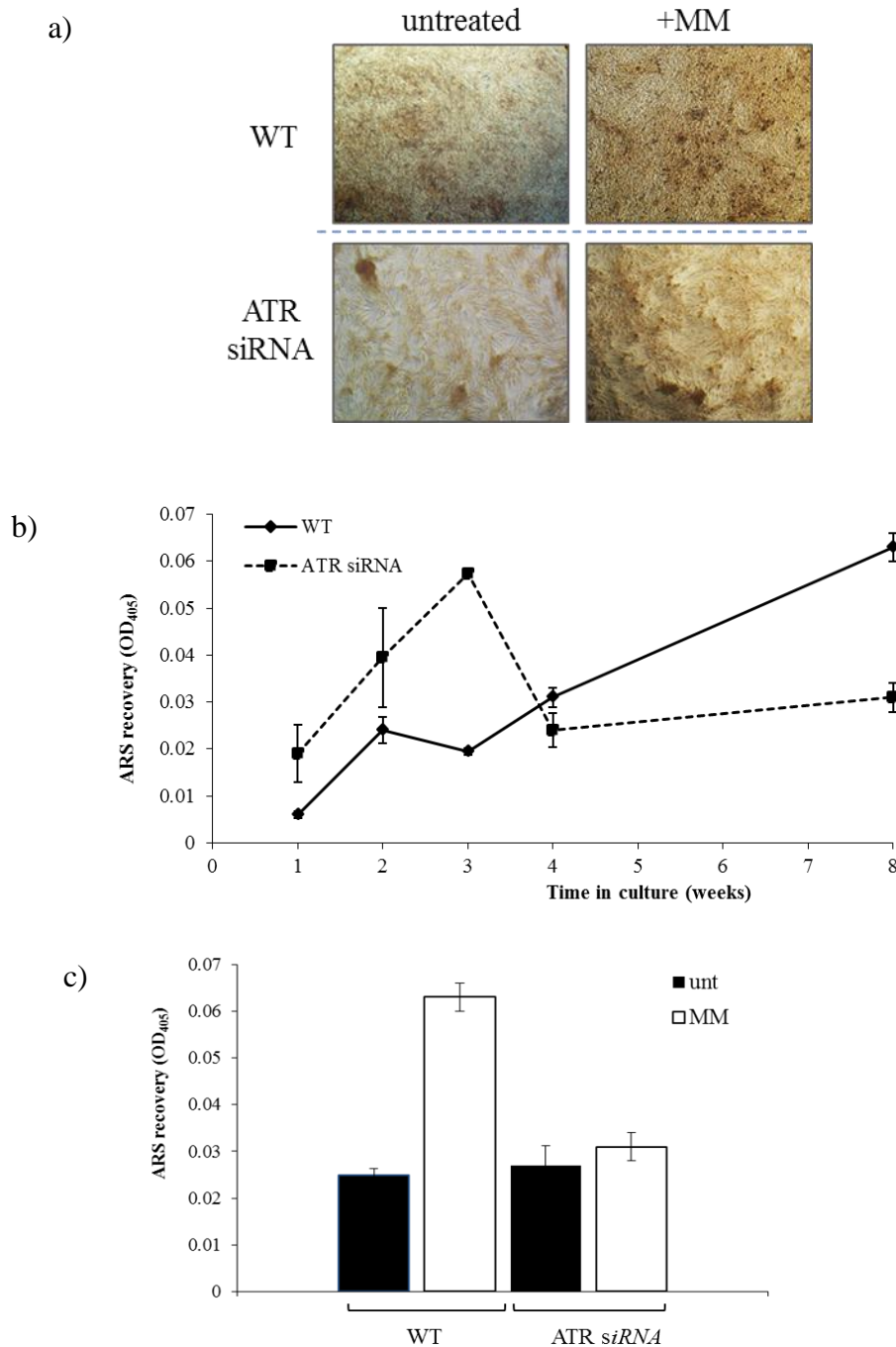


Figure 3.2.3 Bone mineral production in WT and ATR siRNA MG63 cells.

Accumulation of mineral can be observed in MG63 cells after 8 weeks osteoinduction in mineralizing medium (MM) as determined by alizarin red (ARS) staining. **a)** WT and ATR siRNA cells visualized under light microscope after ARS staining after 8 weeks osteoinduction, and **b)** Time-course production of mineral, in the presence of MM, assayed by ARS acid extraction. ATR inhibition did not prevent initiation of mineralization, but appeared to have impacted on the ability to maintain the differentiated status. **c)** ARS quantification of MG63 after 8 weeks osteoinduction, in the presence (MM) or absence (unt) of bone mineralizing medium evidencing a mineralizing problem in the ATR siRNA cells. Data shows the mean variation between 2 replicated cultures, with error bars representing \pm SD.

A cartilage template is required for the formation of the majority of the bones, during endochondral ossification. If chondrogenesis is compromised, it will consequently affect the formation of the skeleton. This could also account for the cartilage deficiencies observed in Seckel syndrome. I therefore investigated specifically the chondrogenesis process in the context of ATR deficiency in an independent model differentiation cell system to determine the requirements for ATR herein.

3.2.2 Chondroinduction of human dermal fibroblasts and the impact of ATR/PCNT deficiency in chondrogenesis.

Fibroblasts and chondrocytes share a mesenchymal origin, and it has previously been demonstrated that fibroblasts can be induced to de-differentiate, and re-differentiate along the chondrocyte lineage, under specific culture conditions when seeded onto a chondrogenic matrix (French et al. 2004). In this environment, the cells lose their elongated fibroblast-like morphology and acquire a spherical phenotype. Dramatic biochemical changes accompany these alterations in morphology. This is evidenced by inhibition or total loss of synthesis of proteins characteristic of an undifferentiated mesenchymal cell phenotype (e.g. type I collagen), and increased synthesis of chondrocyte-specific markers, such as type II collagen, SOX9 and aggrecan. In a further attempt to gain insight into the potential role of defective ATR-function in disrupting normal skeletal development in Seckel syndrome, the role of ATR-signaling during chondrogenesis was investigated.

Clinically relevant primary fibroblasts derived from an ATR-Seckel syndrome patient (FO2-92) as well as PCNT-Seckel (ASB) patient-derived cells were used in this study. Mutation in PCNT is the most common cause of MPD and is also associated with functional ATR-pathway defects. We developed and characterized an *in vitro* chondroinduction system for human dermal fibroblasts, based on French *et al.* (French et al. 2004). To induce chondrogenic differentiation, the cells were seeded at high density on aggrecan-coated wells (see materials and methods), and chondrogenic differentiation was monitored over time. Chondroinduction in culture is associated with agglomeration of single cells into

large aggregates upon exposure to the chondrogenic matrix. When seeded on the aggrecan matrix, both WT and patient cells formed aggregates that grew in suspension in the culture medium, as early as 2h after plating (Figure 3.2.4a). A first indication of a potential defect in chondroinduction in the ATR-S and PCNT-S cells arose with the observation that the Seckel cell aggregates appeared to be generally smaller than the WT. Measurement of the aggregate sizes confirmed this observation (Figure 3.2.4b).

Because chondrogenesis involves exit from the cell cycle, and maintenance of the hypertrophic state, cells need to efficiently arrest proliferation during chondroinduction. Considering ATR's role in cell cycle checkpoint activation and DNA replication, I sought to determine whether chondroinduction in the context of ATR-Seckel Syndrome resulted in ordered cell cycle exit and maintenance of this exit. Analysis of Retinoblastoma protein (Rb) phosphorylation (Figure 3.2.4c) revealed that the cells exit the cell cycle as expected for normal commitment to the chondrogenic pathway. Both WT and patient-derived ATR-S fibroblasts stop proliferating as early as 15hrs after induction, suggesting commitment to chondrogenic differentiation. This tells us that any phenotypes observed are uncoupled from ATR's role in normal DNA replication.

Synthesis of sulphated glucosaminoglycans (GAGs) is a typical marker of chondrocytic cells (Matsubara et al. 1992). Here, our dermal fibroblasts start producing chondrocyte-specific GAGs when cultured in specific concentrations of aggrecan (Figure 3.2.5a). Interestingly, significantly reduced synthesis of GAGs by the ATR-S cells was observed after aggrecan induction. These results suggest that ATR-S cells do not undergo chondroinduction as efficiently as WT fibroblasts, and are consistent with smaller aggregate size seen in these cells upon chondroinduction.

Using semi-quantitative RT-PCR, we analyzed the expression of other chondrocyte-specific markers (Figure 3.2.5b). The increased expression of *SOX9* observed in the WT is consistent with chondro-differentiation and cartilage formation. In both ATR-S and PCNT-S however, *SOX9* expression was not induced, or to a much lesser extent. Conversely, collagen type I transcript levels decrease after aggrecan treatment, consistent with the acquisition of a non-fibroblastic phenotype. We see this in the WT fibroblasts at 48h after chondroinduction, but not as clearly in ATR-S and PCNT-S cells. These results indicate that critical aspects of chondrocyte differentiation are compromised in Seckel syndrome patient derived cells.

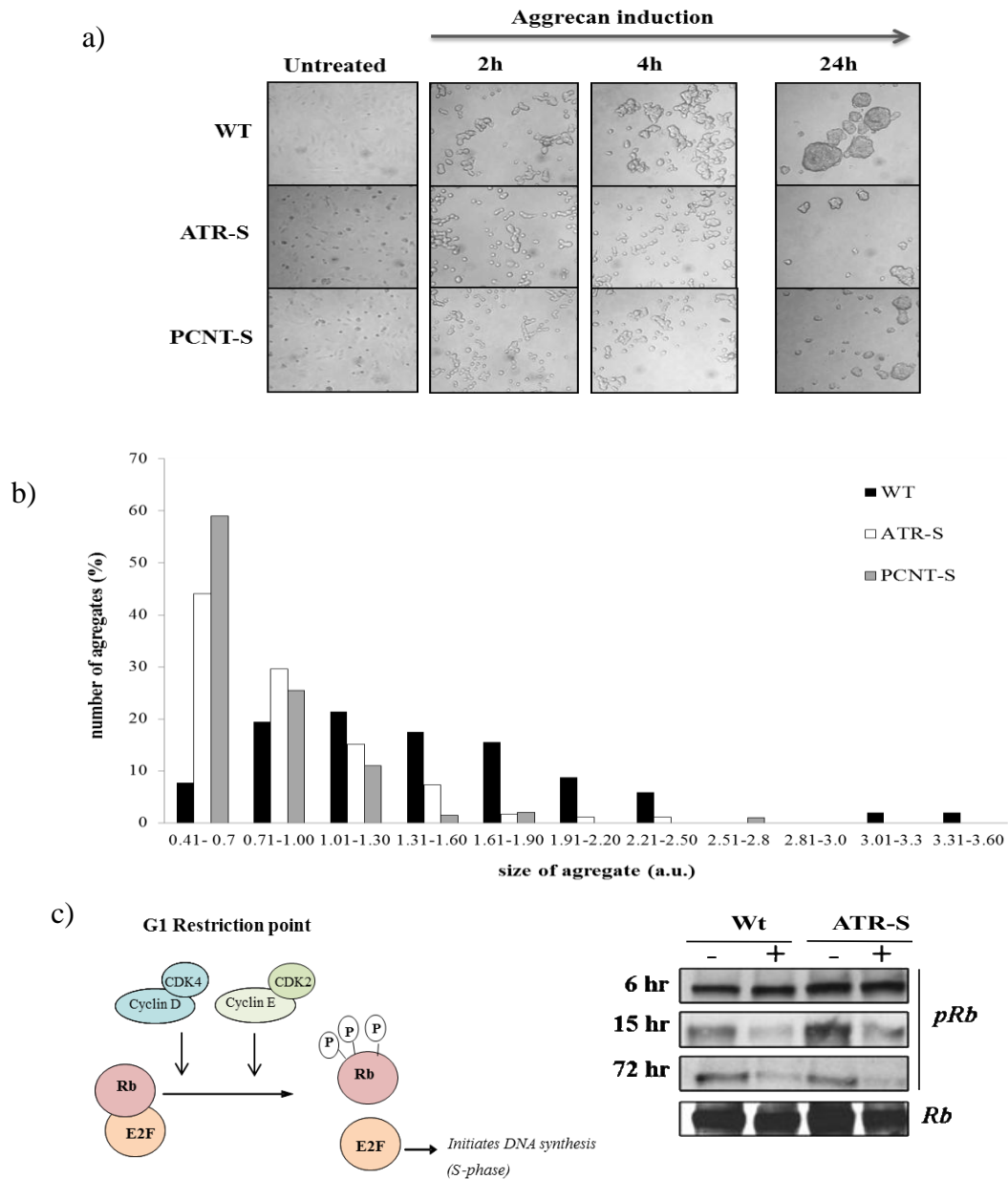
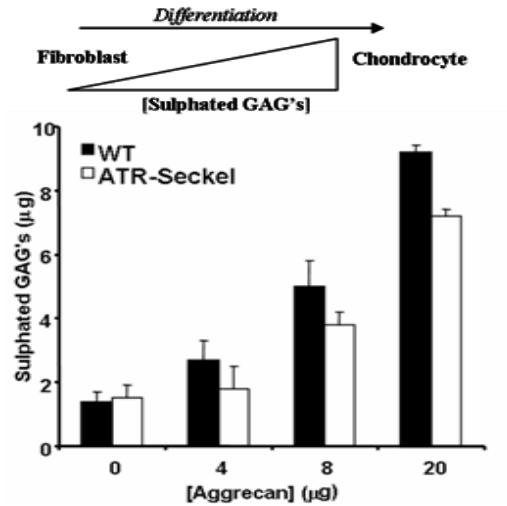


Figure 3.2.4 Aggrecan-induced chondroinduction of human dermal fibroblasts.

a) Phase contrast images (40X) of WT (1BR.3), ATR-S (FO2-98) and PCNT-S (ASB) dermal fibroblasts untreated and at 2h, 4h and 24h following addition to aggrecan coated plates. **b)** Size distribution (arbitrary units = a.u.) of aggregates from WT, ATR-S and PCNT-S fibroblasts following 24hr micromass culture in aggrecan coated plates (n=350 aggregates scored per line) measured using Adobe Photoshop from images acquired on the Zeiss Axiovert platform. Larger aggregate size was a feature of control fibroblasts following chondroinduction compared to ATR-S and PCNT-S patient derived fibroblasts. **c)** Phosphorylation of Retinoblastoma protein (pRb) detected by Western-blot revealed that the cells arrested proliferation following chondroinduction as early as 15hrs following chondroinduction, as indicated by the loss of pRb signal. Both WT and ATR siRNA cells exited with identical kinetics.

a)



b)

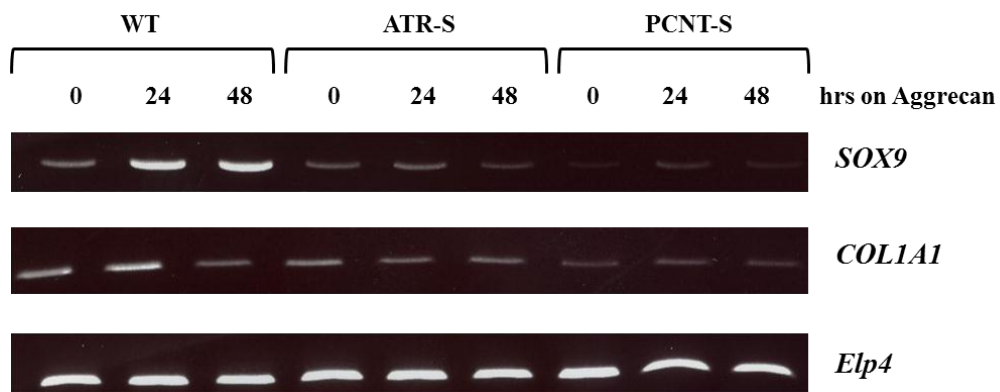


Figure 3.2.5 Expression of chondrocyte-specific markers in ATR-S and PCNT-S patient fibroblasts following chondroinduction.

Chondrocyte differentiation is accompanied by glycosaminoglycans (GAGs) synthesis which can be quantified by dimethylmethylene blue (DMB) assay. **a)** Lower levels of GAGs are synthesized by ATR-S cells growing on aggrecan when compared to WT cells (assay performed previously by Emily Outwin). **b)** *SOX9* expression increases during chondroinduction, whereas type I collagen (*COL1A1*) represents a negative marker as differentiated chondrocytes secrete a specific extracellular matrix. Consistent with this, *SOX9* levels increased while *COL1A1* levels decreased in WT cells during chondroinduction. In contrast, *SOX9* was not induced in either ATR-S or PCNT-S nor did *COL1A1* levels appeared to decrease. Total RNA was extracted after the appointed times and *COL1A1* and *SOX9* expression was detected by semi-quantitative RT-PCR. The expression the housekeeping gene *Elp4*, component of RNA PolIII holoenzyme, was used as an input control for RT-PCR.

To further confirm that the phenotypes observed using the patient cells were due to *ATR* and *PCNT* deficiency, we inhibited *ATR* or *PCNT* by siRNA in WT fibroblasts (Figure 3.2.6a) and tested the expression of chondrogenic markers in these cells by quantitative RT-PCR (Figure 3.2.6). Expression of *COL1A1* is not as efficiently inhibited following chondroinduction after *ATR* as well as *PCNT* knockdown, when compared to WT. This data further reinforces my patient-line derived data suggesting a potential impairment of effective chondroinduction when *ATR* and *PCNT* are limiting.

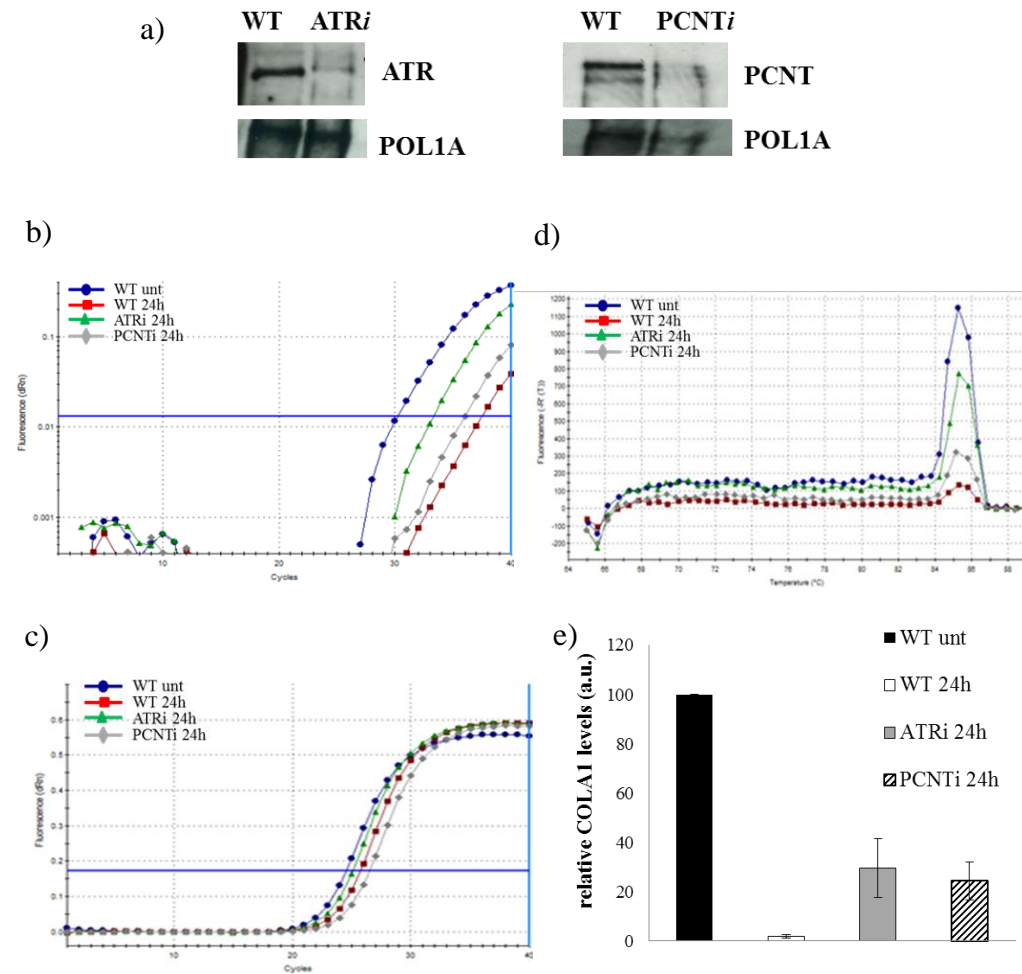


Figure 3.2.6 Analysis of COL1A1 mRNA levels after ATR and PCNT knockdown following chondroinduction by qRT-PCR.

a) siRNA mediated *ATR* and *PCNT* knockdown in WT (1BR.3) fibroblasts was confirmed by western-blot. POL1A (DNA polymerase α p180) levels were used as control for protein loading. 24h after siRNA, the cells were seeded on aggrecan coated plates to induce chondrogenesis and harvested 24h later. The synthesized cDNA was used in quantitative PCR reactions designed to amplify 200bp regions of the *COL1A1* and *GAPDH* gene, using the Qiagen Quantifast SYBR Green PCR kit. Amplification plots were produced by measuring the SYBR green fluorescence after each amplification cycle. A representative amplification plot for *COL1A1* **b)** and *GAPDH* **c)** is shown. **d)** Example of a dissociation curve of the PCR product formed for *COL1A1*, produced by increasing the temperature of the reaction step-wise from 55°C to 95°C and measuring fluorescence at each increase. The experiment was repeated 3 times with samples prepared in triplicate. **e)** ΔC_t values were calculated as specified in materials and methods as averages for the three acquisition runs. *GAPDH* mRNA was used as a reference mRNA. Mean values \pm standard deviations of the means are shown. Consistent with semi-quantitative RT-PCR significantly elevated levels of *COL1A1* were detected in *ATR* and *PCNT* siRNA cells compared to WT 24h following chondro-induction. This is consistent with impaired chondro-induction in these lines.

3.2.3 HIF-1 α /VEGFA expression in ATR-S and PCNT-S cells during chondroinduction

In vivo, the chondrocytic growth plate upon expansion ultimately becomes hypoxic (Araldi et al. 2010). An important chondrogenic transcription factor is the hypoxia inducible factor, HIF-1 α , which induces the expression of a number of genes including *VEGF*. VEGF promotes vascularization enabling the recruitment of osteoblasts and osteoclasts to the growth plate (Zelzer et al. 2004; Maes et al. 2012). I examined HIF-1 α stabilization/expression in control, ATR- and PCNT-Seckel syndrome fibroblast following chondroinduction. I found that chondroinduction induced expression of HIF-1 α in WT cells, under normoxic conditions (Figure 3.2.7b). Interestingly, I repeatedly observed that aggrecan-induced HIF-1 α expression appeared to be significantly delayed/reduced in the ATR-S as well as in the PCNT-S cells compared to WT. This suggests that impaired ATR function may have an impact on the induction of HIF-1 α expression in the context of chondrogenesis. During normal bone development HIF-1 α -induced VEGF expression occurs so as to recruit a blood supply to the epiphyseal growth plate thereby recruiting osteoblasts onto the cartilage template (Cramer et al. 2004). Using semi-quantitative RT-PCR, I found that VEGF expression also increases after aggrecan induction in the WT cells, but is not induced in the ATR-S and PCNT-S cells (Figure 3.2.7c). This observation further suggests a major problem in regulation of the hypoxia response in ATR/PCNT deficient cells, during chondrogenesis. If Seckel cell chondrocytes cannot properly induce VEGF expression they may not be able to efficiently recruit blood supply to the site of bone formation, which ultimately could greatly impair skeletal development via restriction of the growth plate.

Hypoxia also results in increased phosphorylation and consequent activation of the serine/threonine kinase AKT and p38 mitogen activated protein kinase (MAPK), with consequent HIF-1 α stabilization. AKT is required for regulation of skeletal development, involving the mTOR pathway (Rokutanda et al. 2009). PI3K/AKT signaling also promotes survival and synthesis of chondrocytic matrix in the adult cartilage. Mice lacking both *Akt1* and *Akt2* genes show severe growth deficiency and retarded endochondral ossification (Peng et al. 2003). Evidence of AKT inhibition has also been found in human osteoarthritic cartilage (Chen et al. 2012).

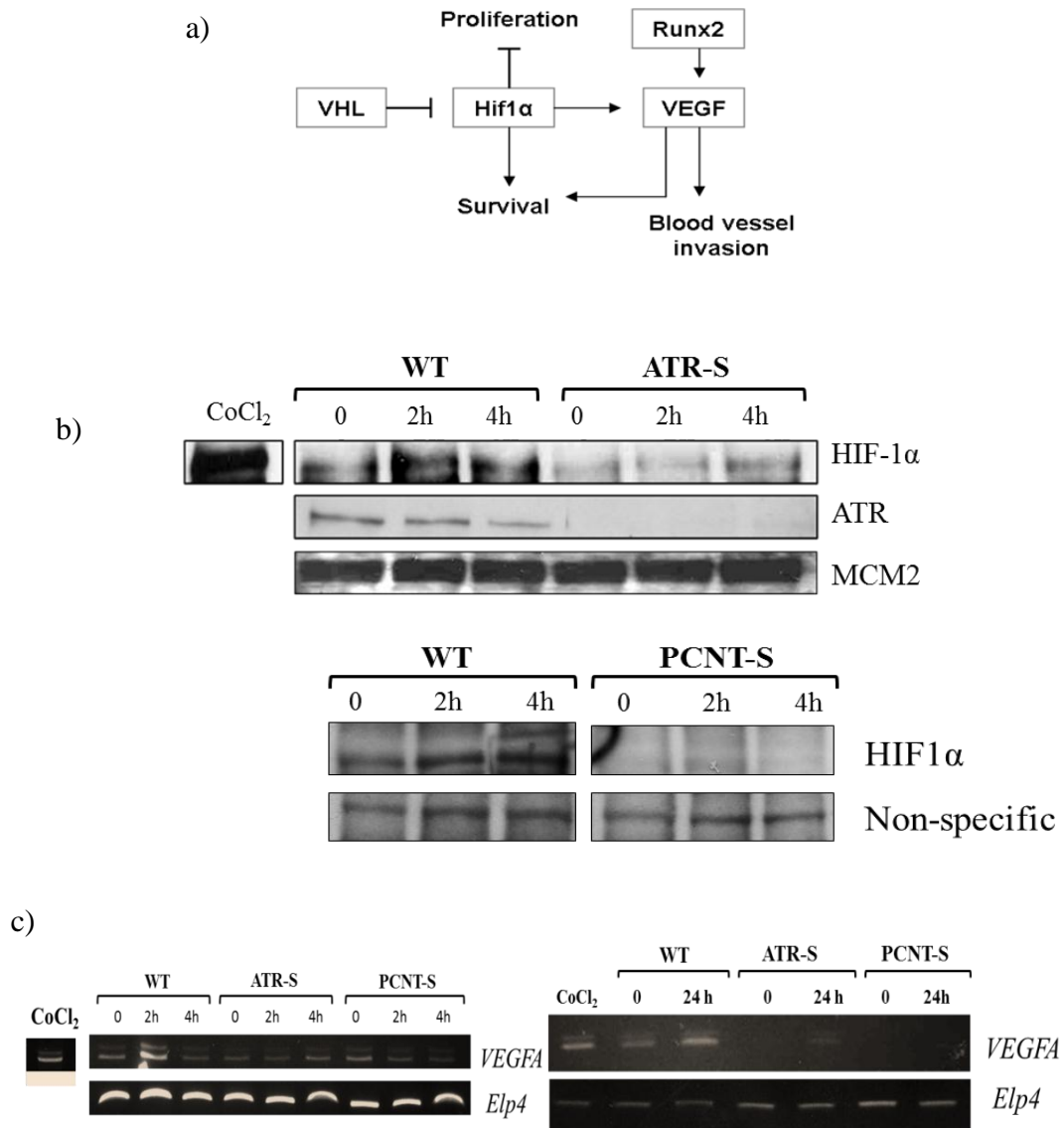


Figure 3.2.7 ATR and PCNT Seckel syndrome fibroblasts exhibit impaired expression of the hypoxia-inducible factor, HIF1 α , and VEGFA after chondroinduction.

a) In the fetal growth plate, HIF-1 α controls *VEGF* expression in the hypoxic areas. **b)** The total levels of HIF-1 α were detected by Western blotting at the indicated times after exposure to the chondrogenic matrix. ATR-S and PCNT-S cells showed reduced induction of HIF-1 α expression, compared to WT, under these normoxic conditions. HIF-1 α and HIF-1 α -dependent *VEGFA* expression by cells treated with CoCl₂ (150 μ M; 24h) was used as a positive control. The expression of MCM2 protein, a component of the helicase complex involved in DNA replication, was used as a loading control. **c)** Using semi-quantitative RT-PCR, control fibroblasts showed enhanced levels of two *VEGFA* transcript isoforms (isoforms *a* and *c*) post addition to the chondrogenic matrix, which is not observed in both ATR- and PCNT-defective cells. The expression the housekeeping gene *Elp4*, component of RNA PolIII holoenzyme, was used as input control for RT-PCR.

I found AKT phosphorylation is induced in this *in vitro* chondroinduction system (Figure 3.2.8c) as early as 4h following aggrecan induction. AKT activation on Thr308 is clearly visible in WT cells. Interestingly I found attenuated AKT activation in ATR-S cells during chondroinduction suggesting that another important programmed chondrogenic event does not occur in these cells (Figure 3.2.8a). AKT is activated by phosphorylation at Thr308 by PDK, which is downstream of PI3K activation. To confirm this AKT activation upon chondroinduction is PI3K dependent, I used a specific PI3K catalytic inhibitor, PI-103. Early chondroinduction markers, HIF-1 α and *VEGFA* expression, were not induced in this system in the presence of the PI3K inhibitor (Figure 3.2.8b,c). This suggests an important role for this pathway in regulating the hypoxia response during chondrogenesis. AKT is particularly important in mediating signaling via the insulin receptor. Additionally, insulin has been found to stimulate chondrogenic differentiation and activate Hif-1 α in normoxia, through the PI3K pathway (Stiehl et al. 2002).

In the next chapter I will investigate the impact of ATR/PCNT deficiency in the cellular response to insulin and its potential impact in the process of chondro-differentiation by examining activation of the PI3K/mTOR pathway in our Seckel Syndrome patient derived cells.

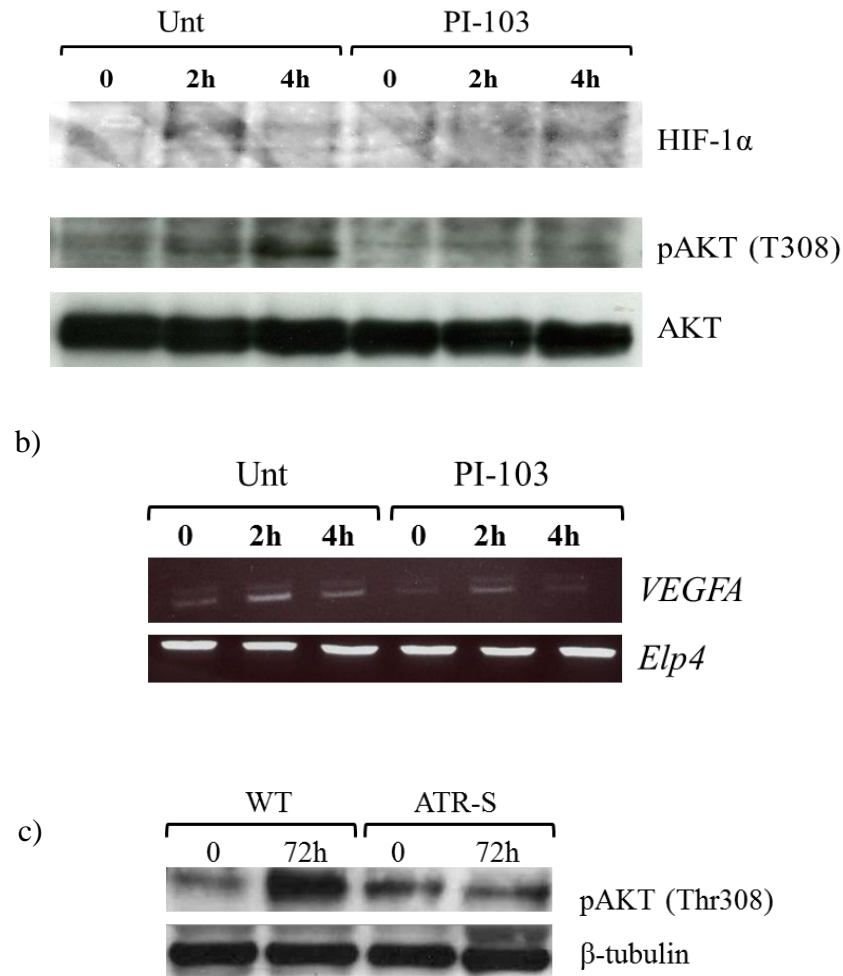


Figure 3.2.8 AKT phosphorylation is not induced following chondroinduction of ATR-S cells, and in the presence of a PI3K inhibitor.

Inhibition of PI3Kinase prevents induction of chondrogenic markers following aggrecan-induced differentiation. **a)** Western-blot analysis of cell extracts following short-term chondroinduction reveals both HIF1 α expression and AKT phosphorylation are not induced in the presence of the PI3Kinase inhibitor, in WT fibroblasts. **b)** Expression of *VEGFA* is inhibited in WT cells after treatment with 150nm of the PI3K-specific inhibitor, PI-103, as determine by semi-quantitative RT-PCR. The expression the housekeeping gene *Elp4*, component of RNA PolII holoenzyme, was used as an input control for RT-PCR. **c)** Increased AKT phosphorylation after 72h chondroinduction was observed in WT cells but not in ATR-S cells. Protein extracts were analyzed by Western-blot using anti-phospho Thr308 of AKT antibody, and β -tubulin for protein loading control.

3.2.4 ORC1 Meier-Gorlin syndrome and IFT43 Sensenbrenner syndrome fibroblasts exhibit impaired chondrogenesis.

Recently, mutations in genes involved in replication licensing were identified in Seckel Syndrome (SS) and/or Meier-Gorlin Syndrome (MGS) patients. These include the origin recognition complex (ORC) proteins ORC1, ORC4, and ORC6, as well as pre-replication complex (pre-RC) proteins CDT1 and CDC6 (Bicknell et al. 2011; Guernsey et al. 2011; Bicknell et al. 2011).

Both disorders, together with Majewski osteodysplastic primordial dwarfism (MOPD) type II, share overlapping clinical features that include accentuated microcephaly, severe intrauterine growth retardation and postnatal growth delay (Majewski and Goecke 1982; Hall et al. 2004). Various degrees of skeletal abnormalities are also observed such as delayed bone age, ivory epiphysis, clinodactyly, brachydactily. MGS is also referred to as “ear, patella, short stature syndrome” (EPS), due to the characteristic hypoplastic or absent kneecaps (patellae) and small ears (the outer ear is a cartilaginous tissue) (Gorlin 1992). Deficiency in origin licensing proteins has also been shown to inhibit the formation of primary cilia in fibroblasts (Tom Stiff, University of Sussex). The primary cilium plays a central role in skeletogenesis, in part by mediating Indian Hedgehog and Wnt signaling (Serra 2008; Haycraft et al. 2008). Skeletal defects are also commonly observed in ciliopathies, as exemplified by Sensenbrenner Syndrome (cranioectodermal dysplasia), a disorder caused by defects in the intraflagellar transport (IFT) machinery (Arts et al. 2011). Although impaired replicative capacity may contribute to the disease phenotype, these associations prompted me to consider the impact of impaired origin licensing machinery in the context of chondrogenesis.

In this section the efficiency of ORC1-deficient MGS and IFT-43 defective Sensenbrenner Syndrome fibroblast to undergo chondroinduction, was investigated. The size distribution of chondroinduction derived-aggregates of both ORC1-defective MGS fibroblasts and IFT43-defective Sensenbrenner Syndrome fibroblasts was smaller than those of control wild type (WT) cells after 24hrs culture on the chondrogenic matrix (Figure 3.2.9a). However, synthesis of glycosaminoglycans (GAGs), a chondrocyte ECM component, was similar in both ORC1 and IFT43 defective cells, comparable to wild type levels (Figure 3.2.9b).

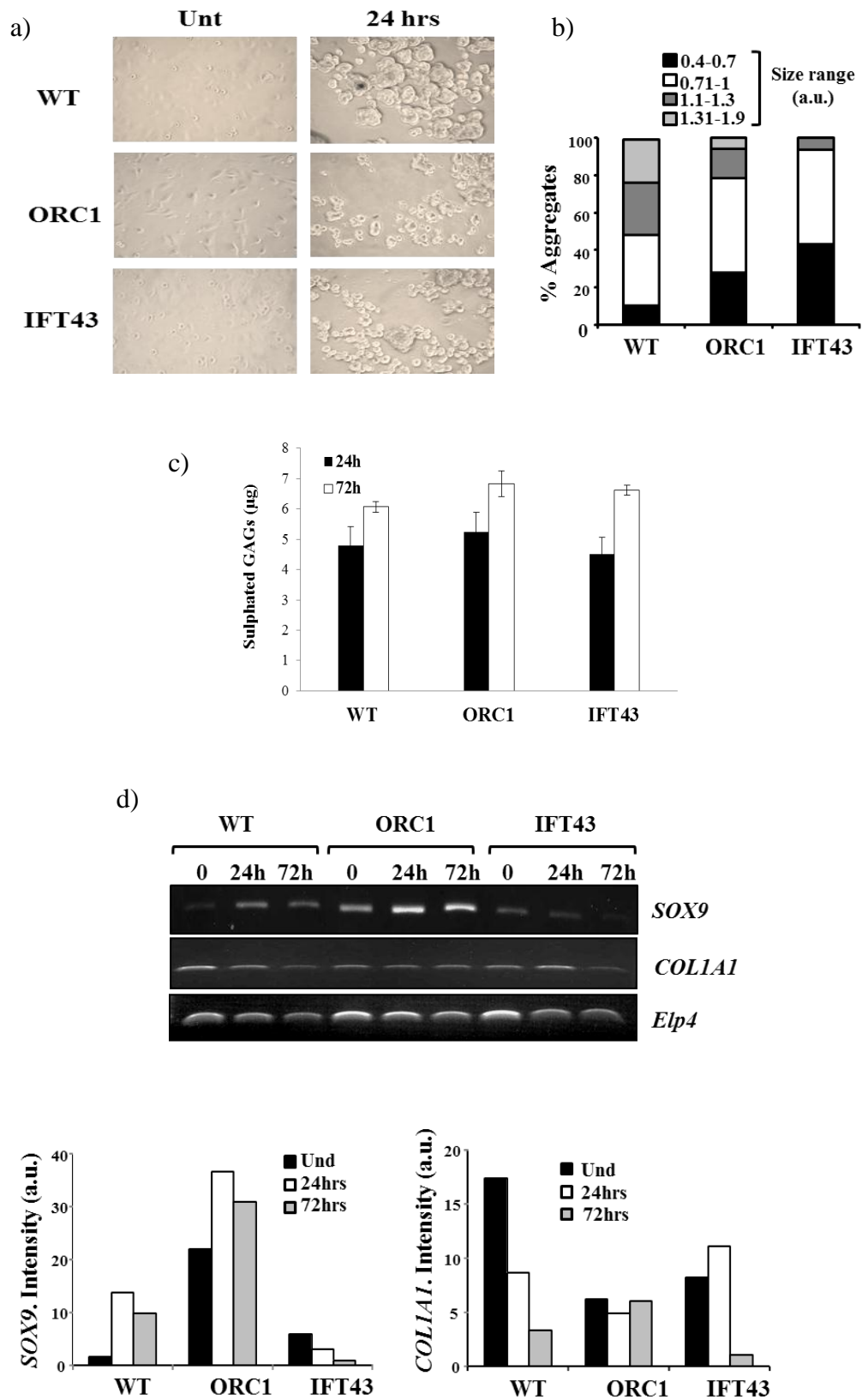


Figure 3.2.9 ORC1 Meier-Gorlin syndrome and IFT43 Sensenbrenner syndrome fibroblasts exhibit impaired chondrogenesis.

a) Phase contrast images (40X) of control (WT) hTERT, ORC1-deficient MGS (ORC1) and IFT43-mutated Sensenbrenner (IFT43) patient-derived fibroblasts at 0 hr and 24hr following addition to aggrecan coated plates. **b)** Size distribution of aggregates from WT, ORC1 and IFT43 fibroblasts following 24hr micromass culture in aggrecan coated plates (n=350 aggregates scored per line) measured using Adobe Photoshop from images acquired on the Zeiss Axiovert platform. Larger aggregate size was a feature of control fibroblasts following chondroinduction compared to ORC1 and IFT43 cells. **c)** Quantification of glycosaminoglycans (GAGs) synthesis by dimethylmethylene blue (DBM) assay showed no difference between levels of GAGs produced by WT or ORC1/IFT43 cells. *SOX9* expression increases during chondroinduction, whereas type I collagen (*COL1A1*) represents a negative marker as differentiated chondrocytes secrete a specific extracellular matrix. **d)** *SOX9* levels increased while *COL1A1* levels decreased in WT cells during chondroinduction. Total RNA was extracted after the appointed times, and collagen type I and Sox9 expression was detected by semi-quantitative RT-PCR. The expression the housekeeping gene *Elp4*, component of RNA PolIII holoenzyme, was used as an input control for RT-PCR.

COL1A1 expression was also analyzed by semi-quantitative RT-PCR. In WT cells I observed a marked decrease in expression of this marker, consistent with chondrogenic differentiation, which is also noticeable in IFT-43 cells. ORC1 MGS cell, however, fail to inhibit *COL1A1* expression to the same extent under these conditions (Figure 3.2.9c). *SOX9* is also a target of HIF-1 α and a key regulator of chondrocyte differentiation. I examined *SOX9* expression in these fibroblasts upon chondroinduction although in this case following 24hrs and 72hrs exposure to the chondrogenic matrix (figure 3.2) using semi-quantitative RT-PCR. *SOX9* expression was found to increase after 24hrs in WT cells. Unexpectedly, aberrantly elevated levels of *SOX9* were detected in the ORC1-defective MGS cells in the un-induced state. The IFT43-defective Sensenbrenner syndrome cells also exhibited some *SOX9* expression in the un-induced cells and could not further induce or indeed maintain expression of *SOX9* upon chondroinduction.

Optimal HIF-1 α expression was observed in control cells within 2hrs after exposure to the chondrogenic matrix (Figure 3.2.10a).

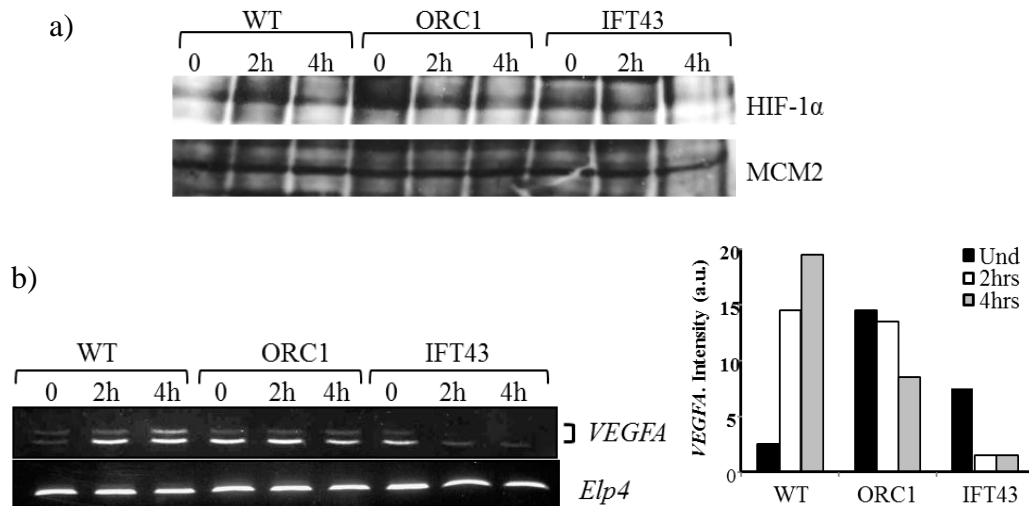


Figure 3.2.10 HIF-1 α and VEGF induction in ORC1 Meier-Gorlin syndrome and IFT43 Sensenbrenner syndrome fibroblasts after chondrogenesis.

a) HIF-1 α levels were quantified by Western blotting at the indicated times after exposure to the chondrogenic matrix. The expression of MCM2 protein, a component of the helicase complex involved in initiation of DNA replication, was used as a loading control. **b)** Differentiated chondrocytes secrete factors that promote vascularization such as *VEGFA* enabling recruitment of osteoblasts to form the trabecular bone. *VEGFA* secretion was measured by semi-quantitative RT-PCR up to 4 hrs after induction. ORC1 deficient fibroblasts show elevated basal levels of *VEGFA* but no increase up to 4 hrs unlike the WT cells, in which a marked increase in *VEGFA* expression is observed. We observed a decrease in *VEGFA* expression in IFT43 fibroblasts. The expression the housekeeping gene *Elp4*, component of RNA PolII holoenzyme, was used as an input control for RT-PCR.

Interestingly, both ORC1-defective MGS and IFT43-defective Sensenbrenner syndrome cells appeared to have constitutively elevated levels of HIF-1 α prior to induction relative to WT, but failed to maintain this expression of HIF-1 α upon chondroinduction. To understand the potential significance or consequences of these differences in HIF-1 α stabilization kinetics, we examined expression of *VEGFA* (gene encoding VEGF) under these conditions, since it represents an important HIF-1 α transcriptional target during chondrogenesis. Using semi-quantitative RT-PCR a clear induction of *VEGFA* isoform *a* was noted in WT fibroblasts within 2-4hrs of exposure to the chondrogenic matrix. Both the *VEGFA* isoform *c* (faster migrating band) and isoform *a* (slower migrating band), were detected. Interestingly, both ORC1-defective MGS and the IFT43-defective Sensenbrenner Syndrome cells appear to exhibit *VEGFA* isoform *c* expression even in the un-induced cells, which, particularly for the Sensenbrenner syndrome cells,

was not maintained upon chondroinduction. *VEGFA* expression (isoform *c*) was not induced above background in the ORC1-defective MGS fibroblasts nor was isoform *a* amplified to a comparable extent as in WT cells.

It was noticeable that both ORC1-defective MGS cells and the IFT43-defective Sensenbrenner syndrome cells differed from control fibroblasts on four endpoints examined as part of the chondroinduction characterisation (aggregate size, HIF-1 α stabilization, *VEGFA* and *SOX9* induction), but defective ORC1 also differed from IFT43 deficiency. ORC1-defective MGS fibroblasts expressed significant levels of HIF-1 α , *VEGFA* and *SOX9* even in the un-induced cultures suggestive of a possible inappropriate engagement with this differentiation process.

Using qRT-PCR to more closely monitor *COL1A1* transcript levels, I observed elevated levels in control fibroblasts, partially decreased at 24h following addition to chondrogenic matrix and reduced to one fifth of the initial level in control WT cells by 72h after chondroinduction. In contrast, ORC1- and IFT43-deficient cells endogenous *COL1A1* levels were not decreased at 24h and less substantially decreased at 72h (2 to 2.5 fold decreased for ORC1-deficient cells) when compared to the WT cells (figure 3.2.11). Additionally, *SOX9* expression as detected by qRT-PCR was shown to increase in WT fibroblasts 24h following aggrecan stimulation. In the ORC1 deficient cells, the pre-induction state already shows high levels of *SOX9* transcript, whereas IFT43 cells, by contrast, showed reduced *SOX9* levels, which only moderately increase following chondroinduction, under the same conditions.

During endochondral bone formation, premature differentiation of immature chondrocytes can retard growth plate expansion and result in skeletal abnormalities (smaller bones) (Farquharson and Jefferies 2000). A transgenic mouse model of ORC1L-patient mutations would be the most appropriate means to formally validate this possibility. In summary, following chondroinduction, in our model culture system for chondrogenesis, both ORC1-defective MGS patient-derived fibroblast and IFT43-defective Sensenbrenner syndrome cells appeared distinct to those of control fibroblasts. This is consistent with aberrant inappropriate unregulated premature chondrogenic differentiation in these patient-derived cell lines that ultimately is not maintained and provides some evidence for the first time that chondrogenesis may be impaired/altered in these conditions. This highlights a novel link between defects in the origin licensing component ORC1 and programmed

differentiation with specific regard to MGS, and IFT with regard to Sensenbrenner Syndrome.

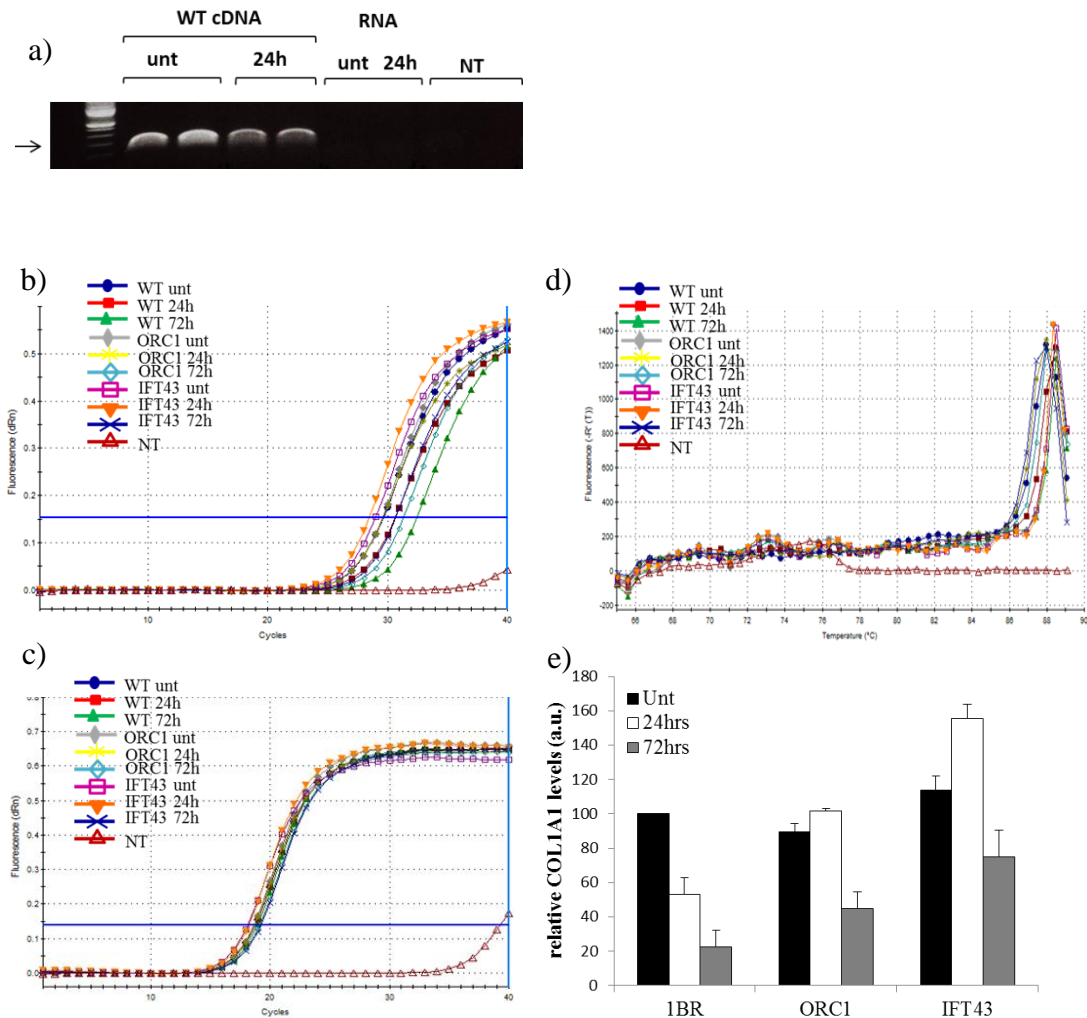


Figure 3.2.11 Analysis of COL1A1 mRNA levels in ORC1 and IFT43 patient cells following chondroinduction, by qRT-PCR.

PCR reactions were carried out using primers designed to amplify regions of the *COL1A1* gene **a)** or the *GAPDH* gene, using cDNA as a template. The arrow indicates formation of a 200bp PCR product. To check for contamination with genomic DNA, a reaction using the RNA material as template was included, as well as a no-template reaction to check for primer contamination. The synthesized cDNA was used in quantitative PCR reactions designed to amplify 200bp regions of the *COL1A1* and *GAPDH* gene, using the Qiagen Quantifast SYBR Green PCR kit. Amplification plots were produced by measuring the SYBR green fluorescence after each amplification cycle. A representative amplification plot for *COL1A1* **b)** and *GAPDH* **c)** is shown. **d)** Example of a dissociation curve of the PCR product formed for *COL1A1*, produced by increasing the temperature of the reaction step-wise from 55°C to 95°C and measuring fluorescence at each increase. The experiment was repeated 3 times with samples prepared in triplicate. **e)** ΔC_t values were calculated as specified in materials and methods as averages for the three acquisition runs. *GAPDH* mRNA was used as a reference mRNA. Mean values \pm standard deviations of the means are shown.

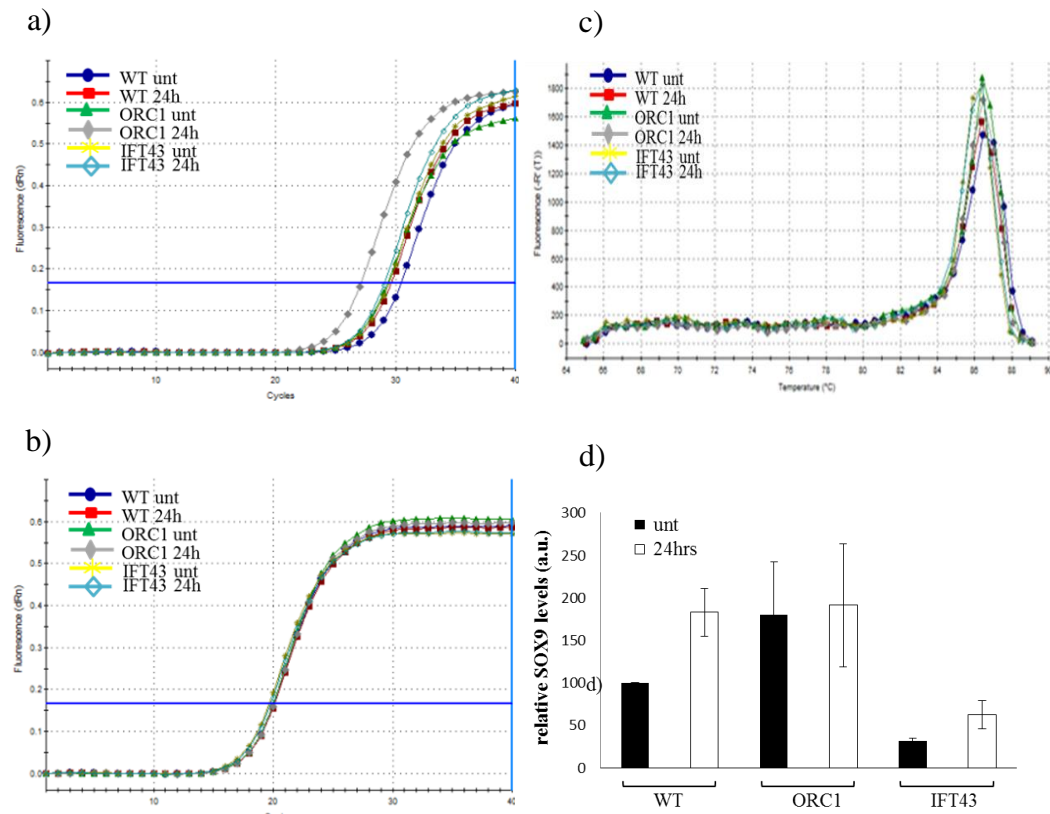


Figure 3.2.12 Analysis of SOX9 mRNA levels in ORC1 and IFT43 patient cells following chondroinduction, by qRT-PCR.

PCR reactions were carried out using primers designed to amplify regions of the *SOX9* gene or the *GAPDH* gene, using cDNA as template. A representative amplification plot for *SOX9* **a)** and *GAPDH* **b)** is shown. **c)** Example of a dissociation curve of the PCR product formed for *SOX9*, produced by increasing the temperature of the reaction step-wise from 55°C to 95°C and measuring fluorescence at each increase. The experiment was repeated 3 times with samples prepared in triplicate. **d)** ΔC_t values were calculated as specified in materials and methods as averages for the three acquisition runs. *GAPDH* mRNA was used as a reference mRNA. Mean values \pm standard deviations of the means are shown.

3.3 Summary

Severe growth retardation and skeletal defects are key clinical features of MPD, including Seckel syndrome, which can be caused by mutations in ATR, as well as PCNT, associated with defective ATR-pathway function. ATR is one of the apical regulators of the DDR, but novel substrates for ATR (and ATM) have been identified in response to DNA damage, which are not directly involved in the DDR network.

In view of the disproportionate impact of impaired ATR signaling on the skeletal tissue, exemplified by Seckel syndrome, I investigated if ATR function could be specifically required during skeletogenesis. Initiation of osteogenesis was not compromised after ATR inhibition, but it had a potential impact on maintenance of the differentiated status. Cartilage tissue is required as a template for bone development, and therefore defects in chondrogenesis directly impact on the skeletal phenotype. Chondroinduction in patient derived ATR-S and PCNT-S cells was less efficient than in control WT cells, as assessed by several parameters (aggregate formation, synthesis of GAGs, expression of chondrocyte-specific markers). Significantly, HIF-1 α and *VEGFA* expression was not induced in ATR-S and PCNT-S cells during chondroinduction, suggesting a serious problem in the regulation of the hypoxia response in these cells. Similarly, activation of PI3K/AKT signaling, required for chondrocyte formation, was not efficient in Seckel patient cells.

Finally, I provided evidence that ORC1-deficient Meier-Gorlin Syndrome (MGS), characterized by microcephaly and skeletal defects, is associated with aberrant chondroinduction. Similarly, an IFT43-defect, such as seen in Sensenbrenner syndrome, also impacts chondroinduction. This data suggests a role for ORC1 and IFT43 in chondrogenesis.

3.4 Discussion

Striking growth retardation and skeletal defects, along with microcephaly, are the key clinical features of the Microcephalic Primordial Dwarfism (MPD) Seckel Syndrome. A hypomorphic mutation in *ATR* was the first identified causative mutation behind this disorder (O'Driscoll et al. 2003). Mutations in the gene encoding the centrosomal protein pericentrin (*PCNT*) also have been shown to cause SS (Griffith et al. 2008). *PCNT* mutations have also been described in microcephalic osteodysplastic primordial dwarfism type II (MOPDII) a related MPD associated with a more marked skeletal involvement and asymmetric dwarfism. More recently, mutations in *ORC1*, *ORC4*, *ORC6*, *CDT1* and *CDC6*, involved in origin replication licensing, have also been identified in Seckel Syndrome and MGS patients (Bicknell, Walker, et al. 2011; Guernsey et al. 2011; Bicknell, Bongers, et al. 2011), suggesting a link with defective DNA replication and these disorders presentations. Seckel Syndrome is a genetically heterogeneous condition associated with defective ATR signaling pathway function, even when the causative mutation is not in *ATR per se* (Alderton et al. 2004). Studies have been done to enlighten the relationship between these mutations and the specific phenotypes observed in the patients. Microcephaly has been proposed to result from a limiting pool of neuroprogenitor stem cells as a result of 1) limited expression and/or 2) elevated apoptosis during brain development, as a consequence of imbalanced regulation of mitosis during neurogenesis (Mochida and Walsh 2001). *PCNT* defects potentially affecting spindle orientation could result in an imbalance between symmetric versus asymmetric stem cell divisions. Not only misregulation of the balance between progenitor/differentiating cells in the neuroepithelium could contribute to microcephaly, but also diminution of total cellularity could explain the extreme short stature observed in primordial dwarfism. The ATR-Seckel mouse model proposes that limited ATR function results in accumulation of DNA damage due to replicative stress during embryogenesis (Murga et al. 2009). The consequent increase in apoptosis during development could account for the clinical features observed in the human patients, as well as in the ATR-Seckel mouse. Additionally, conditional ATR deletion in adult mice was shown cause depletion of a high percentage of proliferating cells in multiple tissue systems (Ruzankina et al. 2007).

ATR and ATM are the canonical DNA damage response apical regulators. However, a high number of new substrates for ATR/ATM phosphorylation after DNA damage have been identified, and some of these are not elements directly involved in the DDR network (Matsuoka et al. 2007). This observation potentially extends the range of cellular processes regulated by these kinases.

As such, and in view of the seemingly disproportionate effect of impaired ATR signaling on skeletal tissues, as observed in Seckel Syndrome, the question arose of whether ATR function could be involved in other aspects of different cellular processes, and specifically if ATR function is required for the formation of the skeleton during human development.

Here, I showed that ATR inhibition in MG-63 osteosarcoma cells did not prevent initiation of osteoinduction, as indicated by the increase in expression of bone specific markers osteocalcin and collagen type I following osteo-induction. However, the ability to maintain the differentiated status did appear to be compromised following *ATR* silencing.

In this osteoinduction model system, some level of cell death was observed following initiation of differentiation, in both WT and ATR siRNA cells. In the mineralizing conditions however, the ATR siRNA cell cultures seemed to result in relatively increased cell death. This observation was not further investigated in this study, but could be a potential point of interest, as pathological loss of bone mass defines the osteoporosis condition and an aged phenotype (associated with osteoporosis) was observed in the conditional ATR knockdown in adult mice (Ruzankina et al. 2007).

A cartilage template is used in the process of formation of the majority of the bones in the skeleton (Karsenty 2008), and as such a defect in chondrogenesis could directly result in an impaired bone formation/skeletal phenotype, as seen in SS.

The impact of ATR signaling function on the process of cartilage formation was investigated using patient-derived fibroblasts from ATR-S and PCNT-S individuals. We optimized here a chondroiduction model system for de-differentiation of human fibroblasts, based on a murine cell model previously described. Some indicators suggested that chondroinduction is less efficient in ATR-S and PCNT-S cells, in this system. The acquisition of a chondrocyte specific round-shaped cell morphology and formation of aggregates in suspension was observed following induction, with both

SS cell types forming markedly smaller sized aggregates when compared to control cells.

Differentiation was accompanied by synthesis of chondrocyte specific factors such as sulphated glycosaminoglycans and the chondrogenic transcription factor *SOX9*, as well as suppression of fibroblastic markers like collagen type I. Notably, both ATR-S and PCNT-S derived fibroblasts did not acquire a chondrocyte specific phenotype as efficiently as the WT fibroblasts, suggestive of a problem in cartilage differentiation in these cells. And importantly, these observed phenotypes are uncoupled from the role of ATR in DNA replication, as the cells were shown to have exited the cell cycle appropriately upon chondro-differentiation. These observations lend me to propose an impact of ATR depletion in aspects of cartilage formation which are not associated with replicative stress.

This observation was further strengthened by siRNA mediated inhibition of ATR as well as PCNT, in WT fibroblasts, which resulted in a less efficient inhibition of collagen type I expression, compared to control cells, following chondroinduction.

Another important aspect during chondrocyte differentiation in the fetal growth plate is the cellular response to hypoxia observed in that environment (Araldi and Schipani 2010). Hypoxia responsive elements are required to promote chondrocyte survival. In my system, I found chondroinduction induced normoxic expression of HIF-1 α in fibroblast cells, after 2-4h post-induction. Moreover, HIF-1 α expression was delayed/attenuated in the SS patient cells, both ATR-S and PCNT-S. This effect was further observed even at 24h after chondroinduction. The small diameter of the cell aggregates does not suggest that a hypoxic microenvironment can be found in the core of the WT chondrogenic aggregates, but this hypothesis needs be further investigated. Expression of the *VEGF* angiogenic factor, a downstream effector from HIF-1 α , was correspondingly induced in WT fibroblasts under the same conditions, but not induced specifically in ATR-S and PCNT-S cells. These combined observations suggest a problem in regulation of the hypoxia response in ATR/PCNT deficient cells, in the context of chondrogenesis specifically.

HIF-1 α can be activated under normoxic conditions, following stimulation with insulin and interleukin-1, and involving activation of the PI3K/AKT pathway (Stiehl et al. 2002). Also, insulin and IGF-1 have a demonstrated role in chondrogenesis, and can induce chondro-differentiation *in vitro* (Phornphutkul et al. 2006).

The PI3K/AKT signaling pathway can be activated by a large number of receptors, including tyrosine receptor kinases such as the insulin receptor and IGF1R (insulin-like growth factor 1 receptor) (Laplane and Sabatini 2012). A number of cellular functions, including cell differentiation, proliferation and survival, are controlled by this pathway in a cell-type specific manner. Chondrocyte hypertrophy and survival during cartilage formation requires PI3K/AKT signaling (Rokutanda et al. 2009). Additionally, some evidence has been found suggesting regulation of PI3K by the cartilage transcription factor Runx2 during this process (Goldring et al. 2006). Cells from aged and osteoarthritic cartilages produce less collagen and cartilage proteoglycan in response to IGF-1, via PI3K/AKT regulation (Chen et al. 2012).

AKT activation is induced following chondroinduction in my model system, in WT fibroblasts. Notably, ATR-S patient cells failed to induce AKT activation under the same conditions. This suggests a major signaling defect involving a key signaling pathway in these cells. Phosphorylation on Thr308 of AKT is a PI3K/PDK-dependent event. Indeed after treatment with a PI3K specific inhibitor, PI-103, chondroinduction failed to induce AKT activation in WT cells. Importantly, other chondrogenic events such as activation of HIF-1 α and VEGF were suppressed after PI3K inhibition. This suggests some crucial chondrogenic events are under PI3K/AKT pathway regulation, and importantly, these are being mis-regulated in ATR-S patient cells.

A direct link between ATR-pathway function and HIF-1 is provided by the fact that the ATR damage-response pathway has been recently shown to be activated by stalled replication forks that result from hypoxia-induced S-phase arrest, involving phosphorylation of H2AX and accumulation of the tumour suppressor p53 (Hammond et al. 2002). Interestingly, oxidative stress has also been shown to inhibit IGF-dependent induction of chondrocyte proteoglycan and collagen, involving changes in regulation of the PI3K/AKT as well as the ERK/MAPK pathways (Starkman et al. 2005).

In order to try and clarify the mechanism by which ATR (and PCNT) deficiencies can have an impact on these seemingly unconnected signaling pathways, I investigated in more detail the PI3K/AKT/mTOR signaling response in Seckel Syndrome patient cells in the next chapter.

Finally, defects in origin licensing proteins cause Meier-Gorlin Syndrome (MGS) and also Seckel Syndrome (Bicknell et al. 2011; Guernsey et al. 2011;

Bicknell et al. 2011). MGS clinical features include microcephaly and bone developmental defects. Skeletal defects are also observed in ciliopathies, namely in Sensenbrenner syndrome (Arts et al. 2011). In order to understand how deficiency in origin licensing proteins may contribute to these specific skeletal phenotypes, I investigated the *in vitro* chondro differentiation of ORC1-deficient MGS, in comparison to IFT-43 deficient Sensenbrenner Syndrome patient cells, which are defective in IFT.

I provide evidence for the first time to suggest that defective ORC1 is associated with aberrant chondro-induction indicating a role for ORC1 in chondrogenesis. Similarly, and for the first time, I provide direct evidence for impaired IFT43 in the context of Sensenbrenner syndrome, as also affecting chondro-induction. With specific regard to ATR, PCNT and ORC1, it should be noted that chondro-induction (and chondrogenesis) involves regulated cell cycle exit. Therefore the impact of these genes upon chondro-induction must be independent of their roles in cell cycle progression. My data provides a cell-based model to help explain the origin of skeletal abnormalities in multiple MPD's (Seckel, MOPDII, MGS) expanding the impacts of defects in these genes.

Chapter Four

*Results II: Insulin-dependent PI3K-AKT-mTOR
signaling in ATR and PCNT-defected patient –
derived cells*

4.1 Introduction

Microcephalic primordial dwarfism (MPD), including Seckel Syndrome, is a disorder of severe global growth impairment associated with profound disproportionate microcephaly. Organism growth and development requires a tightly regulated integration of signals from multiple signaling pathways. Key signaling pathways involved in the regulation of organ and organism size include the receptor tyrosine kinase (RTK)PI3K-AKT-mTOR, and the mitogen-activated protein kinase (MAPK) pathway, controlling aspects such as cell mass, cell proliferation and cell survival through the regulation of ribosome biosynthesis and global protein translation (see Chapter One) (Caron et al. 2010). Growth deregulation is an important aspect of many human diseases, including cancer, and a number of disorders of growth failure or overgrowth are caused by single gene mutations in the RTK-PI3K-AKT-mTOR pathway or the MAPK pathway (Laplante and Sabatini 2012; Tidyman et al. 2009).

The currently known genes mutated in microcephalic primordial dwarfism (*ATR*, *PCNT*, *ORC1*, *CENPJ*, *CEP152*, *ORC1*, *ORC4*, *ORC6*, *CDT1*, *CDC6*, *U4atac*, *IGF-1*) are mainly not components of core growth signaling pathways, but generally involved in cell cycle progression (O'Driscoll et al. 2003; Griffith et al. 2008; Bicknell, et al. 2011; Guernsey et al. 2011; Bicknell, et al. 2011; Netchine et al. 2011). Impaired cell division, resulting in reduced cell number, could be one hypothesis to explain the global growth failure and reduced organism size. However, mutations in *IGF-1* (insulin-like growth factor 1) have also been shown to result in primordial microcephalic dwarfism, evidencing that this specific phenotype is associated with defective regulation of growth signaling pathways (Netchine et al. 2011). Seckel Syndrome is associated with defects in ATR-dependent signaling, even when the mutated gene is not *ATR per se*. (Alderton et al. 2004)

Interestingly, recent studies have found a number of components of the insulin/mTOR signaling pathway are substrates for ATR/ATM phosphorylation after DNA damage (Matsuoka et al. 2007), suggesting functional links between these pathways, although their fundamental biological significance has not been described.

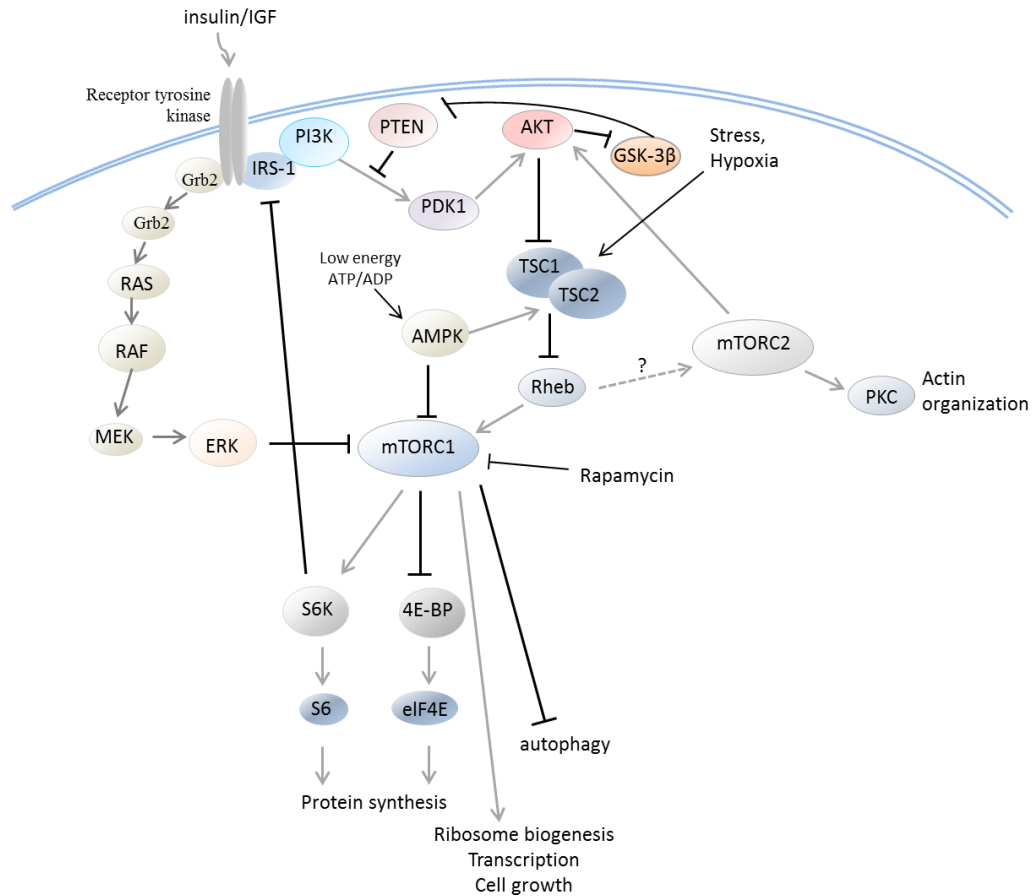


Figure 4.1.1 The complex insulin/mTOR signalling involves negative feedback mechanisms and cross-talk with the PI3K/AKT and RAS/MAPK pathways.

Ligand binding induces phosphorylation of IRS-1 resulting in recruitment of the p110 catalytic subunit of PI3K. AKT phosphorylates and inhibits TSC function, leading to mTOR activation. Through 4E-BP1, rapamycin-sensitive mTORC1 drives cell growth and proliferation by enhancing eIF4E-mediated mRNA translation. S6K1 activation by mTOR leads to IRS-1 phosphorylation and proteasomal degradation.

In this chapter I investigated the insulin-dependent PI3K-AKT-mTOR signal transduction in ATR-defective and PCNT-defective Seckel Syndrome cell lines, to determine whether impairments of this pathway may underlie the severe dwarfism observed in these patients.

4.2 Results

4.2.1 mTORC1-dependent S6K activation is not inhibited by rapamycin in ATR-S and PCNT-S cells

The macrolide rapamycin was first identified as a bacterial anti-fungal agent produced by *Streptomyces hygroscopicus*, identified on Easter Island . It was later found to have both immunosuppressive and cell anti-proliferative properties. Rapamycin (and its derivatives) is currently used therapeutically for its anticancer activity, and as an immunosuppressant for transplant patients (Blagosklonny 2012). The primary receptor for rapamycin in the cell is FKBP. The rapamycin-FKBP complex binds to and inhibits mTORC1 (Yip et al. 2010). Whereas mTORC1 is sensitive to acute rapamycin treatment, mTORC2 is generally considered to be non-responsive to rapamycin. However, prolonged rapamycin treatment may also interfere with mTORC2 function (Sarbasov et al. 2006).

To investigate mTORC1 function in Seckel Syndrome, I exposed patient-derived fibroblasts to short, acute rapamycin inhibition. Treatment with rapamycin results in rapid loss of S6K phosphorylation. While negatively regulating mTORC1 downstream substrates, it has been shown that inhibition of mTORC1-dependent signaling by rapamycin *induces* strong activation of AKT, in a negative feedback mechanism dependent on S6K/IGF-1R/PI3K.

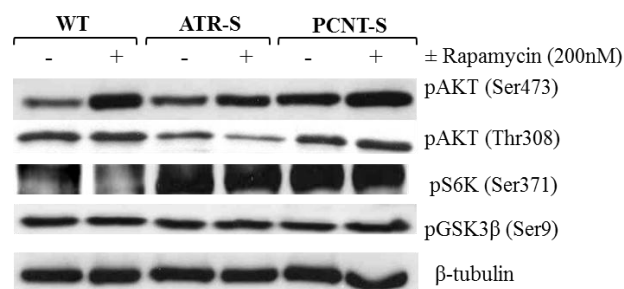


Figure 4.2.1. S6K activation is not inhibited by treatment with Rapamycin, in ATR-S and PCNT-S cells.

WT, ATRS and PCNT-S exponentially growing primary cells were treated with 200mM rapamycin for a period of 4hours. Protein extracts were analysed by Western-blot using phospho-specific antibodies.

As expected, the treatment induced the feedback activation of AKT in the WT cells, and less markedly, also in Seckel patient fibroblasts. Under these conditions however, AKT activation did not impact on phosphorylation of AKT-dependent substrates such as the glycogen synthase kinase-3 β (GSK3 β).

I found rapamycin did not inhibit mTORC1-dependent S6K phosphorylation specifically in the ATR-S and PCNT-S cells unlike WT fibroblasts (Figure 4.2.1). Moreover, the endogenous levels of phosphor-S6K appeared abnormally high in both ATR-S and PCNT-S, when compared to the WT cells, suggesting that mTORC1 may be hyperactive under these conditions. Interestingly, S6K1 defects result in growth retardation.

I then looked at the signaling response to insulin stimulation in ATR-S and PCNT-S cells, as S6K continuous feedback inhibition of IRS-1 could result in insulin insensitivity in these cells. It is possible and predictable that the impact of ATR and PCNT deficiency on mTOR signaling could be mechanistically different, albeit with a similar outcome.

4.2.2. Insulin- dependent activation of mTOR signaling pathway components in ATR-S and PCNT-S cells

Because of the known role of insulin signaling in development and growth, I looked at insulin-dependent mTOR pathway signaling in primary fibroblasts derived from Seckel Syndrome patients, both ATR-defective (FO2-92), as well as PCNT-defective (ASB) cells.

To minimize the contribution of serum-derived insulin, the cells were serum-starved for 72h prior to re-addition of serum and/or insulin. Activation of the mTOR pathway was investigated after re-introduction to normal growing media (15% FBS) or insulin alone. Importantly, physiologically relevant concentrations of insulin (200nM) were used in this study. mTOR pathway signaling response was assessed by looking at the phosphorylation status of a number of components of the pathway. In particular, phosphorylation of S6K is a convenient measure of the activity of mTORC1 branch, and phospho-AKT levels can inform on mTORC2 function.

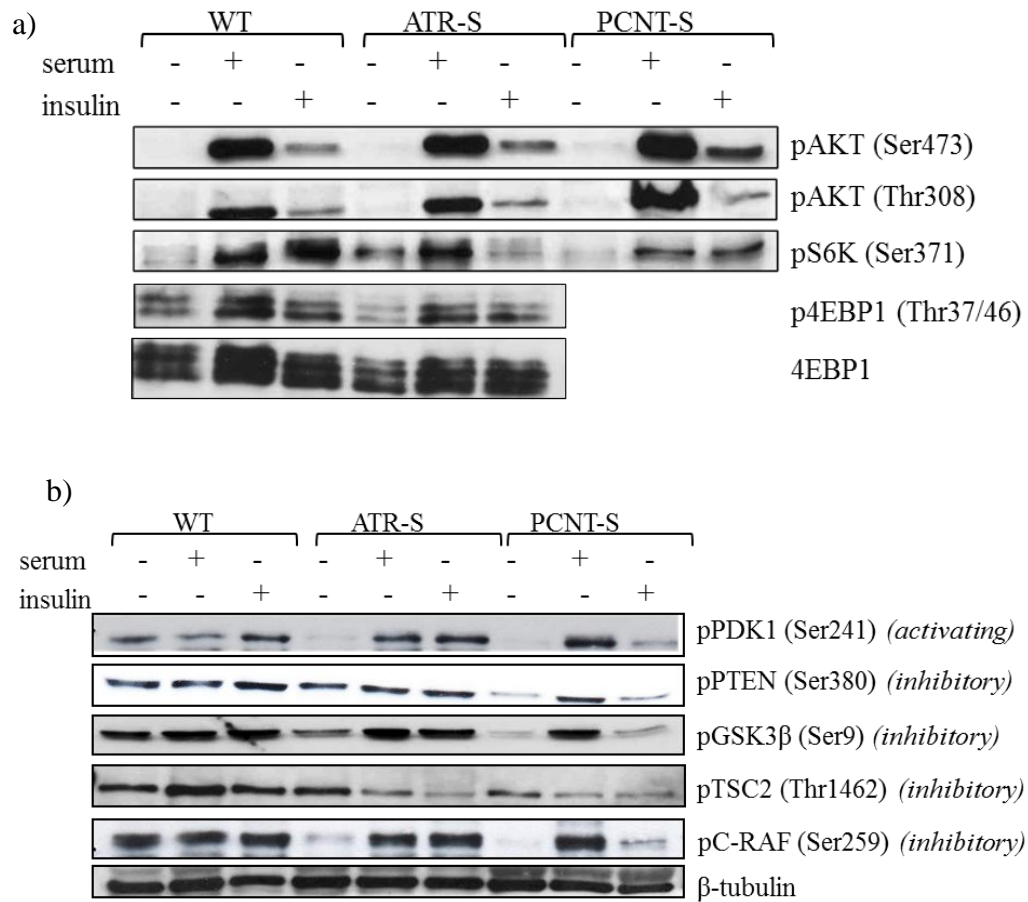


Figure 4.2.2. Activation of PI3K/AKT/mTOR signaling pathway components following serum/insulin stimulation.

WT, ATR-S and PCNT-S primary cells were serum starved for 72h and reintroduced to complete medium (15%FCS) or 200nM insulin for 2h. Protein extracts were analyzed by Western-blot using phospho-specific antibodies. β -tubulin expression was used as protein loading control.

AKT activation is triggered by phosphorylation at two key residues: Thr308 by PDK1, and Ser473 by mTORC2. Serum starved cells showed efficient inactivation of AKT, as expected. Following stimulation, Thr308 and Ser473 phosphorylation on AKT was attenuated after insulin when compared to serum stimulation, the latter being the result of an array of other serum components and growth factors found in the growth media that are also able to induce AKT activation. AKT was efficiently activated in serum deprived cells when reintroduced to full-serum media. Looking at insulin stimulation alone, both WT as well as ATR-S and PCNT-S cells respond to insulin by phosphorylating AKT and the pattern of activation was the same at both phosphorylation sites. However, looking further

downstream at p70S6 kinase (S6K) phosphorylation, a problem involving insulin - dependent S6K regulation in the ATR-S cells becomes evident as they do not respond to insulin specifically (Figure 4.2.2.). Insulin stimulation in the WT cells results in evident S6K phosphorylation, and to a lower extent also in PCNT-S cells, but fails to stimulate S6K activation in the ATR-S cells. In addition, some phosphorylated S6K was still detected in the growth arrested ATR-S cells.

Hyper or prolonged S6K activation results in a negative feedback in the insulin receptor by inhibition of IRS-1 (O'Reilly et al. 2006). This result implies a problem in the mTOR pathway regulation in response to insulin signaling in SS cells, which may lie at the level of the insulin receptor itself or be the result of a break elsewhere in the signal transduction pathway.

Activated mTORC1 phosphorylates 4E-BP1 leading to its release from elf4E, which allows it to form initiation factor complexes and thus activate protein synthesis (Hay and Sonenberg 2004). Interestingly, 4EBP1 is also an ATR/ATM target for phosphorylation following DNA damage (Matsuoka et al. 2007). 4E-BP1 phosphorylation in ATR-S cells seemed to follow a normal induction pattern.

Paradoxically, 4E-BP1 and S6Kinase phosphorylation seemed to be differently affected/deregulated between the ATR and PCNT deficient cells even though they are both the downstream targets of mTORC1 activation. Studies have shown that mTORC1 can have different outputs to S6K and 4EBP1 (X. Wang et al. 2005).

Two different ribosomal S6 kinases, S6K1 and S6K2 act downstream of mTORC1 and phosphorylate the 40s ribosomal subunit S6 (Phin et al. 2003). S6K1 has been associated with cell size regulation (Derheimer and Kastan 2010; Fingar et al. 2002). Interestingly, S6K2 has been found to localize to the pericentriolar area of the centrosome (Burma and Chen 2004; Rossi et al. 2007). Potential disturbance of S6K subcellular localization in pericentrin deficient cells could be involved in its deregulation.

In order to identify where in the insulin/mTOR pathway the signaling cascade is being deregulated, I looked at the activation of a number of key components of this pathway.

PDK1 acts downstream from PI3K, which is recruited to the IRS and activated following insulin stimulation, in a PI3K dependent manner (Bakkenist et al. 2003; Vanhaesebroeck et al. 2000). In both WT and ATR-S cells serum/insulin

stimulation results in PDK1 phosphorylation on Ser241, a necessary step for PDK1 activity. In PCNT-deficient cells, PDK1 is activated in the presence of serum, but less efficient after insulin stimulation. This suggests a problem in an early signaling event in this pathway, in PCNT-S cells specifically.

The protein and lipid phosphatase PTEN (Phosphatase and Tensin Homolog Deleted on Chromosome 10) is a negative regulator of the PI3K/AKT pathway, opposing PI3K-derived PIP3 accumulation. Phosphorylation at Ser380 promotes PTEN stabilization, while inhibiting its phosphatase activity (O'Driscoll et al. 2006; Yang et al. 2012).

PTEN phosphorylation is not induced in PCNT-S cells, following insulin. Under these conditions, in PCNT-deficient cells, PTEN is abnormally active, which may result in suppression of the pathway downstream. Conversely, PTEN inactivation would result in constitutive activation of the PI3K/AKT pathway. This continued effect could eventually lead to insulin insensitivity due to the feedback inhibition of IRS-1 by active S6K. Interestingly, PTEN can localize to the centrosome where it is involved in chromosome stability, DNA repair and cell cycle arrest (down-regulation of cyclin D1) (Riballo et al. 2004; Planchon et al. 2008). The phosphorylation status of PTEN at different sites seems to affect its subcellular localization.

The glycogen synthase kinase 3 (GSK3 β) is negatively regulated by insulin signaling. AKT activation mediated by the IRS-1/PI3K axis leads to phosphorylation of GSK3 β resulting in its inactivation (Grimes et al. 2001). GSK3 β inactivation is impaired in PCNT-S cells following insulin. GSK3 β also directly phosphorylates IRS-1 and impairs insulin signaling and is involved in development of insulin resistance (Kaidanovich et al. 2002). Additionally, GSK3 β was recently found to be a potential regulator of CDC25A (Kang et al. 2008), establishing a direct link to the DNA damage response/cell cycle-regulation machinery.

The tuberous sclerosis complex (TSC1-2) is a negative regulator of mTORC1 (Huang et al. 2008). In response to insulin, activated AKT phosphorylates TSC2 on Thr1462, and inactivates TSC1/TSC2, releasing its inhibition of Rheb, which in turn activates mTORC1. TSC2 inactivation was defective after serum stimulation as well as insulin in both Seckel patient lines. TSC2 can also be phosphorylated by AMPK in response to low intracellular ATP and/or hypoxia (Murga et al. 2009; Inoki et al. 2006; O'Driscoll et al. 2003; Ruzankina et al. 2007; Brugarolas et al. 2004).

Additionally, ERK signaling activates mTORC1, involving TSC1-TSC2 complex phosphorylation by RSK. RSK is also able to phosphorylate TSC2 on the AKT conserved sites, as well as at a novel site (Ser 1798). Interestingly, TSC2 is also an ATR-ATM substrate after DNA damage (Murga et al. 2009; Matsuoka et al. 2007).

c-RAF (or Raf-1) is a serine/threonine kinase mainly implicated in the induction of proliferation via the Ras/MEK/ERK pathway. An inhibitory binding site on c-Raf (Ser259) can be directly phosphorylated by AKT (Murga et al. 2009; Moelling et al. 2002). C-Raf is phosphorylated and inactivated in serum-deprived cells, resulting in proliferation arrest. The AKT-dependent inhibition of c-Raf following serum-deprivation was sustained in WT cells after 2 hours of re-introduction to serum/insulin. Remarkably, serum-starvation did not result in AKT-dependent inactivation of c-Raf in both Seckel cell lines (Figure 4.2.2b). PCNT-S cells failed to inactivate c-Raf after insulin. Interestingly, c-Raf is also an ATR/ATM substrate after DNA damage (Lee et al. 2012; Matsuoka et al. 2007). These results seem to point to another important signal deregulation in Seckel Syndrome cells involving a different signaling cascade.

4.2.3. *ATR* knockdown results in inhibited insulin-dependent activation of AKT and S6K.

A knockdown of *ATR* in an independent cell line, the MG63 osteosarcoma, was used to further investigate the impact of *ATR* deficiency specifically on aspects of mTOR pathway signaling, and to corroborate the phenotypes observed in the patient cell lines. PI3K and PDK1-dependent phosphorylation of AKT on Thr308 did not occur specifically in *ATR* depleted cells, in response to insulin stimulation (Figure 4.2.3.). Correspondingly, phosphorylation of S6K downstream of mTORC1 was also defective in the *ATR* knockdown cells, under the same conditions, when compared to control cells. These results further strengthen the observation that *ATR* deficiency is associated with impairment of insulin-dependent mTOR signal transduction.

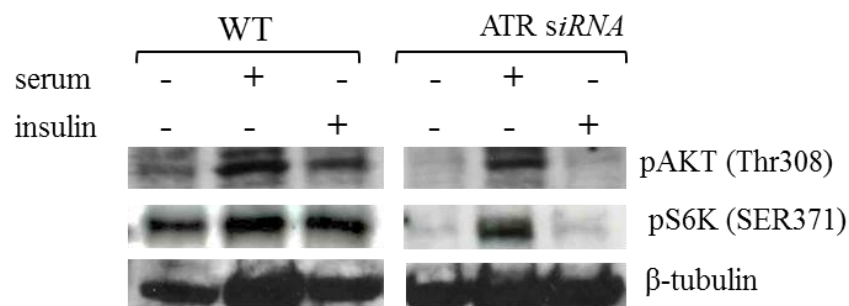


Figure 4.2.3. *ATR* knockdown in MG63 cells inhibits insulin-dependent activation of AKT and p70S6K. Control (WT) and *ATR* knockdown (*ATR* siRNA) cells were serum starved for 72h and reintroduced to complete medium (15% FBS) or insulin (200nM) for 2h. *ATR* siRNA cells failed to efficiently activate AKT, as well as S6K following insulin stimulation. Protein extracts were analyzed by Western-blot using phospho-specific antibodies. β-tubulin was used as a control for protein loading.

4.2.4. Insulin-dependent activation of PDK1 is reduced also in a PCNT-defective lymphoblastoid cell line

An independent PCNT-S lymphoblast cell line (CV1559; affected Seckel/MDPII PCNT 658G>T; p.E220X) was used to investigate aspects of mTOR signaling and confirm some of the observations made using the PCNT-S fibroblast line. These cells also show some level of deregulation in the mTOR pathway, as the PCNT-S lymphoblasts also failed to specifically activate PDK1 following insulin stimulation, thus confirming the previous observations in the fibroblast cells. In PCNT-S cells, the AKT and PDK1 activation profiles suggest deregulation in early events in the insulin/ mTOR signaling cascade (Figure 4.2.4).

Collectively, the phenotypes observed in both Seckel patient derived cells, as well as following ATR knockdown point to an association between defective ATR/PCNT and deregulation of the insulin/ mTOR pathway, a key signaling cascade involved in regulation of cell and organism growth. These novel findings shed new light into the potential contribution of defects in the PI3K-AKT-mTOR pathway to the clinical presentation of these patients. In fact, subsequent careful clinical evaluation of PCNT-defective individuals has shown that they all develop a pronounced insulin-resistant form of diabetes (Huang-Doran et al., 2011).

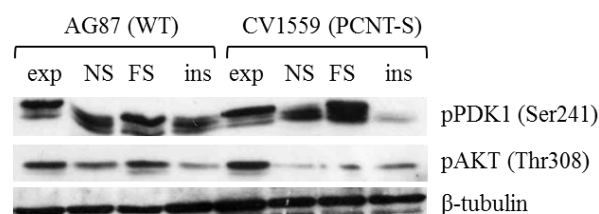


Figure 4.2.4. Insulin stimulation results in PDK1 phosphorylation in control but not in PCNT-S LBLs.

Exponentially growing (exp) control (WT) and PCNT-S (CV1559) lymphoblast cell lines were serum starved for 72h (NS) and reintroduced to complete growing medium (FS) or 200nM insulin (ins) for 2h. Insulin stimulation failed to induce PDK1 phosphorylation in PCNT-S cells, unlike the WT. The pattern of AKT phosphorylation on Thr308 was comparable between WT and PCNT-S lines. Protein extracts were analyzed by Western-blot using phosphor-specific antibodies. β -tubulin was used as a control for protein loading.

4.2.5. PI3K activation is compromised in PCNT-S cells

The PI3K pathway mediates the mTOR response to growth factors. Insulin or insulin-like growth factors (IGFs) binding to their receptors leads to recruitment and activation of PI3K by the insulin receptor substrate (IRS) (Whitehead et al. 2000). IRS-bound PI3K converts phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3). The subsequent PIP3 accumulation results in the phosphorylation and activation of PDK1, which in turns phosphorylates AKT on Thr308. Activated AKT can positively regulate mTORC1 activation by phosphorylating and suppressing TSC1-TSC2, and thus removing its inhibitory effect over mTORC1 (Rokutanda et al. 2009). PI3K activation is therefore an important step in this signal transduction, and therefore represents a key step in the regulation of these phosphorylation events.

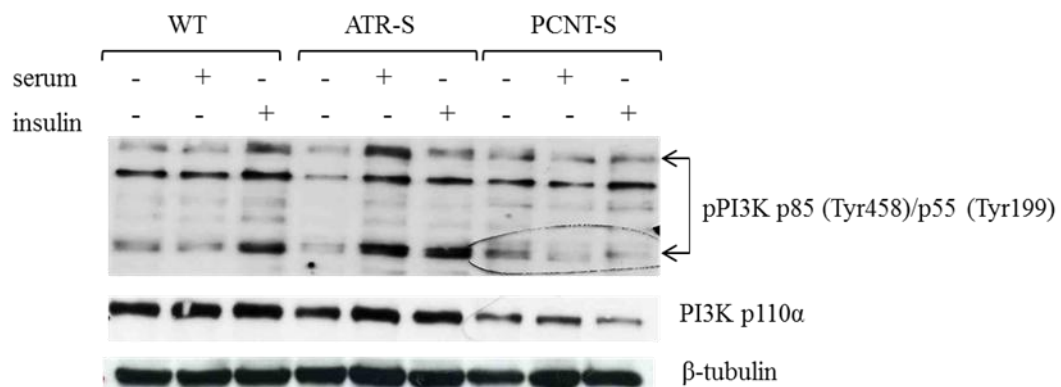


Figure 4.2.5 Insulin/serum stimulation results in PI3K phosphorylation in WT and ATR-S cells, but fails to induce PI3K activation specifically in PCNT-S cells, which showed also reduced levels of PI3K.

WT, ATR-S and PCNT-S fibroblasts were serum starved for 72h and reintroduced to complete medium (15% FBS) or 200nM insulin for 2h. Protein extracts were analyzed by Western-blot using specific antibodies. β-tubulin was used as protein loading control.

Strikingly, both serum as well as insulin stimulation failed to activate PI3K in PCNT-deficient cells, following serum deprivation (Figure 4.2.5). This result was further confirmed in an independent PCNT-defective fibroblast line (data not

shown). Moreover, endogenous levels of PI3K appeared abnormally low in these cells. Comparatively, efficient PI3K activation in response to insulin was observed in the ATR-S line investigated, comparative to WT cells.

A fraction of PI3K has been found to localize to the centrosome (Kumar et al. 2010). At least one study reports a potential association between PI3K and pericentrin, as the PI3K-dependent kinase activity was found to immunoprecipitate with PCNT antibodies in response to insulin (Kapeller et al. 1995).

I wanted to investigate if PI3K is being recruited to the centrosome in PCNT-defective Seckel cells and whether PI3K cellular localization is altered in response to insulin stimulation. Visualization of PI3K by immunofluorescence in the cells did not reveal any change in PI3K localization after induction with both serum and insulin alone (data not shown), which was observed mainly in the cytoplasm, as expected.

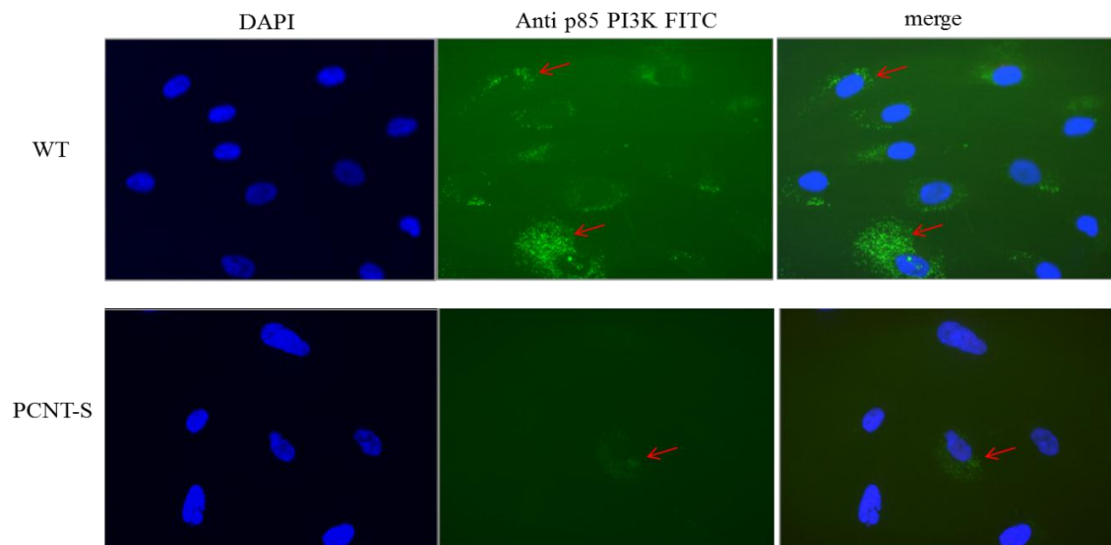


Figure 4.2.6. Subcellular localization of PI3K in WT and PCNT-S primary fibroblast analyzed by immunofluorescence.

Cultured WT and PCNT-S fibroblast cells were fixed, permeabilized and immunostained with the anti-p85PI3K antibody and a FITC conjugated secondary antibody (green). DAPI counterstain (blue) provides nuclear staining. 400X magnification. PI3K is mainly distributed in the cytoplasm. PCNT-S cells show reduced levels of PI3K when compared to WT cells.

However, what was striking in all conditions was the reduced overall level of PI3K detected in the PCNT-S cell line (Figure 4.2.6) when compared to PI3K levels in control WT cells. This could represent a serious defect with potential impact not

only on regulation of mTOR signaling as well as other signaling cascades that operate downstream from PI3K.

To confirm the previously described pericentrin-PI3K interaction, and to investigate this association in the context of pericentrin deficiency, I used a PCNT-S lymphoblast cell line. WT and PCNT-S cells were lysed and immunoprecipitated with either PCNT or PI3K antibodies (p85 and p110 α PI3K subunits). The immunoprecipitates were then analyzed for the presence of PI3K and pericentrin, respectively, by immunoblotting. PCNT was detected in PI3K immunoprecipitates from WT (AG87) cells, thus confirming the interaction between these two proteins (Figure 4.2.7a). As expected, PCNT was not detected in PI3K-immunoprecipitates from the PCNT-S line (CV1559).

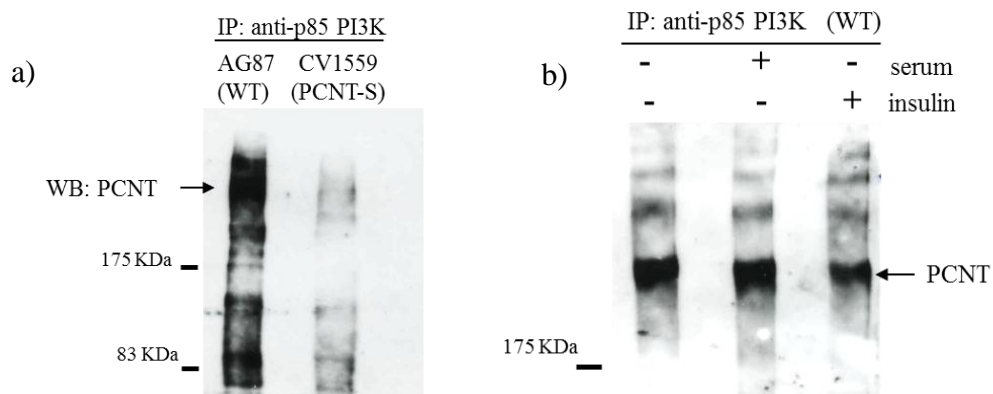


Figure 4.2.7. PCNT immunoprecipitates with PI3K.

a) PCNT detected by western-blot analysis in immunoprecipitates using an anti-p85PI3K antibody in control (AG87) but not in PCNT-S (CV1559) LBLs. b) serum-starvation in does not affect PCNT-PI3K interaction in WT LBLs.

Serum-deprivation, and subsequent stimulation with complete medium or insulin did not seem to affect PCNT-PI3K interaction (Figure 4.2.7.b). However, I was not successful in confirming this interaction in the reverse IP, as we failed to detect PI3K in PCNT immunoprecipitates (Figure 4.2.9). This could be due to different antibody efficiencies for immunoprecipitation, or the fact that only a small fraction of PI3K interacts with PCNT at centrosomes at any one time.

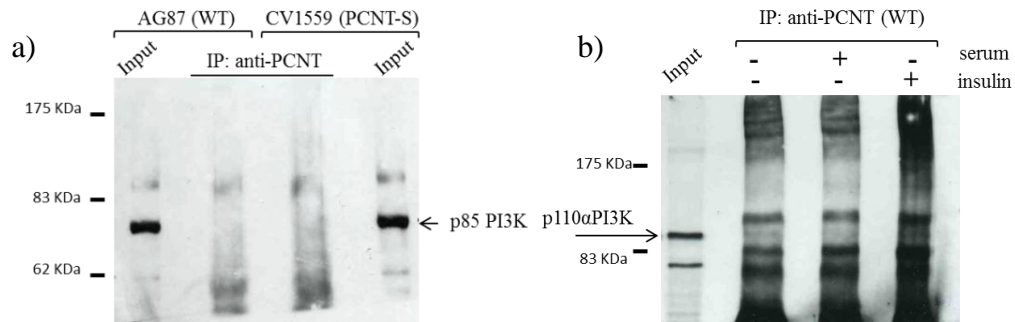


Figure 4.2.8. p85 PI3K is not detected in PCNT immunoprecipitates.

a) p85PI3K not detected by western-blot analysis in immunoprecipitates using an anti-PCNT antibody in control (AG87) and PCNT-S (CV1559) LBLs, both in exponentially growing cells or **b)** following serum-starvation and re-stimulation with serum/insulin. Input (WCE) represents 1/10 of the amount used for the IP.

4.2.5 PI3K levels are reduced following siRNA-mediated *PCNT* knockdown

To confirm that the signaling defects observed were a direct result of pericentrin depletion in the cell lines investigated, a transient siRNA-mediated PCNT knockdown was introduced in C2C12 cells, a mouse myoblast line frequently employed in mTOR pathway evaluations. PCNT expression was successfully reduced to undetectable levels in this cell line (Figure 4.2.7.).

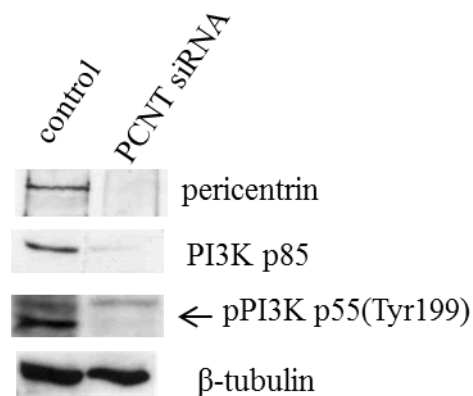


Figure 4.2.9. p85 PI3K is depleted in C2C12 mouse myoblasts after PCNT knockdown.

C2C12 cells were transfected with PCNT siRNA (Dharmacon SMARTpool siRNA). PCNT, p85PI3K and phospho-PI3K (p55/p85) levels in cell lysates were examined by western-blot at 24h after transfection. PCNT knockdown resulted in reduced levels of p85 PI3K, and consequently of PI3K phosphorylation. β-tubulin was used as a control for protein loading.

Importantly, I found p85PI3K levels simultaneously downregulated following PCNT inhibition, suggesting the proteins co stabilize. PI3K downregulation after PCNT depletion is also reflected in decreased PI3K activation as determined by levels of phosphorylated p55(Tyr199)PI3K. I will now use this system to try and confirm the phenotypes observed in the patient cells. These results establish a possible interplay between pericentrin function and PI3K-associated signaling networks, such as the mTOR pathway.

4.3 Summary

MPD, including Seckel syndrome, is a rare disorder defined by extreme growth restriction and severe microcephaly, which has been associated with impaired ATR-pathway function. RTK signaling via the PI3K-AKT-mTOR pathway is essential for cell and organismal growth, promoting ribosome biogenesis and protein translation, and mutations in components of this pathway are linked to growth deregulation (e.g. S6K1 knockout mice show reduced body and cell size). Interestingly, ATR/ATM phosphorylate a number of components of the insulin/mTOR network in response to DNA damage. Moreover, *acanthosis nigricans*, commonly caused by insulin resistance, has been observed in SS, and the ATR-Seckel mouse also exhibits a suppressed growth hormone--insulin-like growth factor 1 axis.

In this chapter I found deregulation of the insulin-dependent PI3K-AKT-mTOR signal transduction in ATR-defective and PCNT-defective Seckel Syndrome cell lines. S6K levels were endogenously elevated in ATR and PCNT-S cells, and unresponsive to rapamycin inhibition. Furthermore, examination specifically of the insulin-dependent signal transduction in these cells revealed impairment/unresponsiveness at various levels. These defects were also observed following ATR knockdown, and in a separate, patient-derived PCNT-S LBL. Importantly, PI3K activation is specifically compromised in PCNT-S cells. The interaction between PI3K and PCNT was confirmed by immunoprecipitation. A compromised insulin-mTOR signal transduction in ATR and PCNT defective cells, could underlie the severe dwarfism observed in these patients.

4.4 Discussion

Microcephalic primordial dwarfism (MPD) is a disorder characterized by severe growth impairment and disproportionate microcephaly. Causative mutations for MPD include the DDR apical kinase ATR, and the centrosomal protein pericentrin (PCNT). ATR pathway function is required for stable DNA replication fork progression and efficient DDR response, including G2/M cell cycle checkpoint control. Defects in centrosomal proteins can result in supernumerary centrosomes, spindle abnormalities, impaired mitotic progression and genomic instability. Symmetric cell divisions are required for stem cell self-renewal. Aberrant spindle orientation could result in an imbalance between symmetric vs asymmetric divisions and thus impair the balance of progenitor cells/differentiating cells. Not only the deregulation of asymmetric divisions in the neuroepithelion could contribute to the observed microcephaly, but the reduced total cellularity could also explain the short stature observed in primordial dwarfism. Notably, both ATR and PCNT defective MPD patient cells exhibit impaired ATR-pathway function. It is therefore likely that these defects should impact specifically on cells and tissues with a high replicative rate, e.g. during neurogenesis.

ATR knockout in the mouse is embryonic lethal. A transgenic mouse model, incorporating the Seckel syndrome hypomorphic ATR-splicing mutation (O'Driscoll et al. 2003) faithfully mimics the Seckel syndrome/MPD human phenotype (Murga et al. 2009). Elevated embryonic replicative stress is seen in the ATR-S mouse, resulting in elevated apoptosis, which could account for the striking growth defect.

However, interestingly, a suppressed insulin-like growth factor-1 (IGF-1) and growth hormone (GH) axis was also observed in the ATR-S mouse model. Additionally, *acanthosis nigricans*, a hyperpigmentation of the skin commonly caused by insulin resistance, has been observed in Seckel syndrome patients, and PCNT-defective MDP exhibit severe insulin resistance and diabetes (Huang-Doran et al. 2011). These observations could have implications for the intrauterine and postnatal growth retardation observed in MDP/Seckel syndrome.

Defects in the insulin-AKT-mTOR axis have been known to underlie major growth defects. Impairment of insulin signaling is particularly important during

development. A severe growth phenotype is seen in *Igf-1* and *Irs* mutant mice, associated with insulin resistance (Tamemoto et al. 1994; DeChiara et al. 1990). As mentioned in the previous chapter, insulin-PI3K-AKT signaling plays a pivotal role in the processes of endochondral ossification and skeletal growth. The association between mTOR pathway dysfunction and growth defects has been well established. AKT is an important mediator of insulin metabolism. *Akt1/Akt2* double *KO* in mice results in dwarfism and skeletal muscle atrophy (Peng et al. 2003). The mTORC1 substrate S6K1 has been associated with cell size regulation, and *S6k* deletion also results in small sized mice (Shima et al. 1998).

A number of components of the insulin-mTOR pathway are novel substrates for ATR and ATM phosphorylation in response to types of DNA damage, although the biological significance of these interactions is yet unknown (Matsuoka et al. 2007).

In view of these observations, and because of the level of cross-talk now anticipated between the DDR and the mTOR network, I investigated the PI3K-AKT-mTOR signaling in response to insulin in ATR and PCNT defective MPD cell lines, to determine whether defects in this pathway could contribute to the severe growth phenotype that results from *ATR* and *PCNT* mutations.

Surprisingly, I found evidence of deregulation of the insulin-dependent-PI3K-AKT-mTOR signal transduction in ATR and PCNT defective MDP lines. Rapamycin inhibition of mTORC1 should result in rapid loss of S6K phosphorylation, and activation of AKT via an S6K-IRS-PI3K-dependent feedback mechanism. S6K levels were endogenously elevated and not inhibited by rapamycin in ATR and PCNT deficient cells, in contrast to WT cells. Examination specifically of the insulin-dependent signal transduction in these mutant cells revealed impairment/unresponsiveness at several points (Figure 4.4.1).

Specifically, insulin-stimulation, after serum-starvation, failed to induce S6K phosphorylation in both ATR-S and PCNT-S cells, suggesting an important problem in the response to insulin in these cells, which could lie at the level of the insulin receptor itself or be the result of a break elsewhere in the signal transduction cascade.

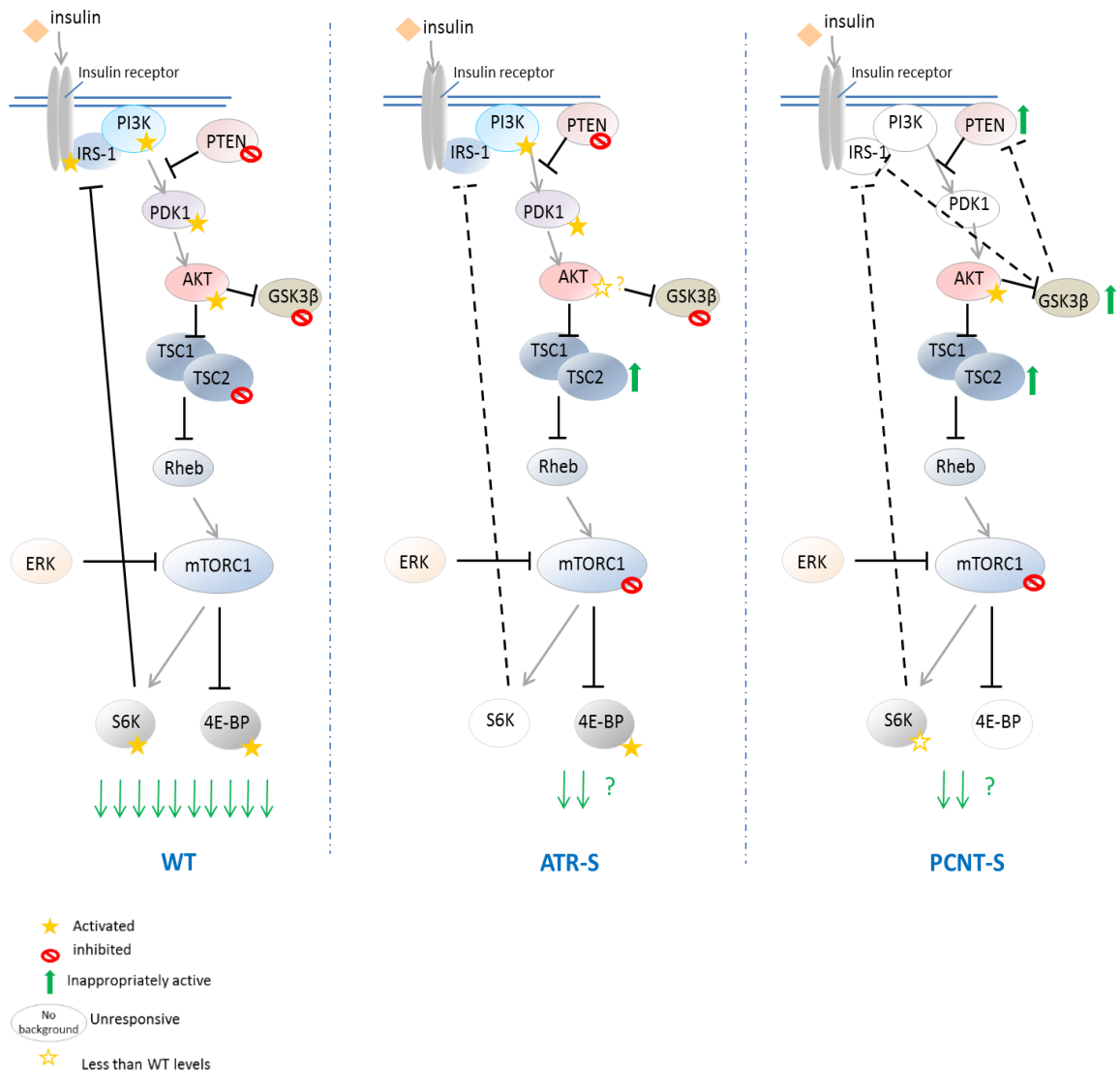


Figure 4.4.1. Schematization of the PI3K-AKT-mTOR pathway activation in response to insulin in WT, ATR-S and PCNT-S cells.

Further interrogation of the pathway by looking at the activation status of key components, suggests that different mechanisms could account for the defect in the insulin response in the ATR and PCNT defective cells.

In the context of ATR deficiency, the TSC1-TSC2 complex is not efficiently inhibited in response to insulin. TSC1-TSC2 is the main upstream negative regulator of mTORC1 function, and hyperactivity of the TSC1-TSC2 complex would result in reduced mTORC1 activation, which seems to be the case in ATR-S cells, indicated by non-responsiveness of S6K to insulin stimulation, and lower phosphorylation of 4E-BP1. S6K phosphorylation was still detected in the growth arrested ATR-S cells (Figure 4.2.2a), and seems to be endogenously elevated in these cells (Figure 4.2.1).

Hyper or prolonged activation of S6K can negatively impact insulin signaling by inhibition of the insulin receptor substrate.

In PCNT-deficient cells, the activation profile of the pathway in response to insulin stimulation is distinct. Insulin addition results in a lower level of activation of S6K in PCNT-S cells, compared to WT. PI3K activation is specifically compromised in PCNT-defective cells, which results also in reduced PDK1 activation. Furthermore, activation of the PTEN phosphatase, which negatively regulates PI3K activity, was also not induced in PCNT-S cells. Phosphorylation, and *inactivation*, of GSK-3 β is also impaired in PCNT-deficient cells. Interestingly, GSK-3 β directly phosphorylates and inhibits IRS-1 and PTEN, and has been involved in the development of insulin resistance. This suggests an upstream, problem in the regulation of insulin response in PCNT deficient MPD cells, compared to ATR-mutated SS cells.

Furthermore, expression of PI3K expression is significantly reduced in PCNT MPD fibroblasts, as observed by anti- p85PI3K immunofluorescence, when compared to control levels in WT cells (Figure 4.2.6). This could represent a serious defect that could also impact on other signaling cascades operating downstream from PI3K. An association between PCNT and PI3K has been reported in the literature. I confirmed the PI3K-PCNT interaction by immunoprecipitation in a WT LBL (Figure 4.2.7). Furthermore, siRNA-mediated PCNT knockdown resulted in reduced PI3K expression (Figure 4.2.9). This represents an important association that could be responsible for the PI3K-dependent signaling defect in PCNT-mutated cells. This could also contribute to the insulin-resistance phenotype, and diabetes, manifested in PCNT-MPD patients.

Insulin-dependent signaling defects were also observed following ATR knockdown in an independent cell line (MG63 osteosarcoma); and also in a distinct patient-derived PCNT lymphoblastoid line, confirming some of the initial observations in ATR and PCNT deficient MPD patient fibroblasts. Interestingly, inhibition of the ATR-related ATM-kinase results in decreased IGF-IR (insulin like growth factor- I receptor) expression, and IGF-IR cDNA expression restores normal radioresistance in AT cells (Peretz et al. 2001).

A compromised insulin-mTOR signal transduction in ATR and PCNT-defective cells could underlie/contribute to the severe dwarfism observed in these patients, and thus constitute a novel pathomechanism for ATR+PCNT-defective MDP.

Chapter Five

*Results III: Defective ATR-dependent DNA
Damage Response in cells from individuals with
primary defects in mTOR pathway function*

5.1 Introduction

In the last chapter I provided evidence of deregulation of insulin-dependent PI3K/AKT/mTOR signal transduction in patient-derived cell lines defective in ATR signaling, establishing a previously uncharacterized relationship between these pathways. Similarly, I demonstrated that ATR defective/impaired signaling compromises such distinct processes as differentiation (namely chondrogenesis, Chapter 3) and insulin/mTOR signaling events at various levels (Chapter 4).

PCNT-defective forms of osteodysplastic primordial dwarfism have been associated with diabetes and insulin resistance (Huang-doran 2011) and early-onset diabetes and *acanthosis nigricans* have been reported in MOPDII patients. Additionally, the ATR-Seckel syndrome mouse exhibits a suppressed Insulin-like Growth Factor-1/Growth Hormone (IGF1–GH) somatotrophic axis (Murga et al. 2009; O'Driscoll 2009). These observations are particularly relevant since several members of the insulin/AKT/mTOR network were recently identified as ATM/ATR substrates in response to DNA damage (Matsuoka et al. 2007), although the significance of this is yet unclear. Nevertheless, this implies that there is an extensive level of crosstalk between these pathways (Figure 5.1.1).

Collectively, these novel findings have implications to the understanding of the particular set of clinical manifestations seen in Seckel syndrome/MOPDII, whose overt clinical features are severe microcephaly and dwarfism.

A role for AKT on cell cycle regulation and checkpoint signaling is already well established (Kandel et al. 2002; Shtivelman, Sussman, and Stokoe 2002; N. Xu et al. 2010). AKT modulates the function of a number of factors involved in normal cell cycle progression, such as increasing activation of c-Myc, Cdk2, and CDC25 phosphatase B, as well as inactivation of the CDK inhibitors p21^{Cip1} and p27^{Kip1} (Xu, 2012 for review) (Figure 5.1.2).

Additionally, the PI3K/PTEN/AKT also has a role in the cellular response to DNA damage. The DDR kinases DNA-PK, ATM and ATR can activate AKT in response to different genotoxic stresses (Lu et al. 2006; Viniegra et al. 2005; Caporali et al. 2008). AKT activation, or constitutively active/overexpression of AKT have been shown to surpass cell cycle checkpoints and promote mitosis in the presence of DNA damage (Kandel et al. 2002). These cells continue to divide despite

exposure to mutagenic agents, and are prone to accumulate mutations (N. Xu et al. 2010), that can lead to neoplastic transformation.

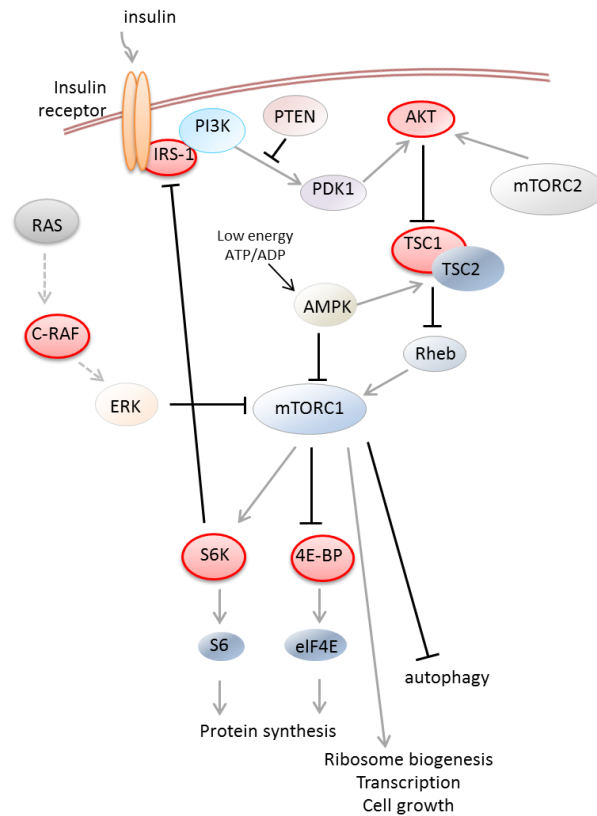


Figure 5.1.1. Insulin-AKT-mTOR pathway signal transduction.

Highlighted in red are some of the novel ATM/ATR substrates identified following DNA damage (Matsuoka et al. 2011).

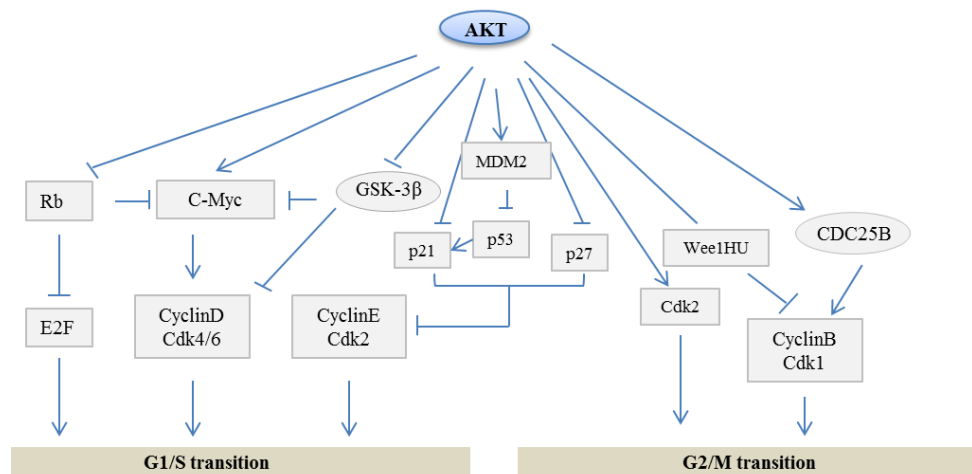


Figure 5.1.2. Involvement of AKT in cell cycle progression.

The AKT kinase modulates the activity of proteins associated with cell cycle regulation. (adapted from Xu et al.2012)

The checkpoint kinase CHK1, an essential regulator of the G2/M transition, is directly phosphorylated by AKT on Ser280, resulting in increased cytoplasmic localization of CHK1. This effect results in *inhibition* of the checkpoint, and has been observed in breast cancers associated with increased AKT activation and reduced PTEN (Puc et al. 2005; Barré and Perkins 2007). Importantly, AKT activating and/or *PTEN* inactivating mutations are frequently found in a number of cancers, promoting tumor proliferation and survival. Specifically, *AKT3* mutations are very common in malignant melanoma and *PTEN* mutations in breast and ovarian cancers. Furthermore, activation of AKT has been associated with resistance to chemotherapy in breast and ovarian cancers.

Microcephalic primordial dwarfism, including Seckel Syndrome and MOPD typeII are associated with profound growth retardation and microcephaly. Interestingly, these clinical features are also seen in disorders of the mTOR pathway, an example of which is Donohue Syndrome (DS). To further probe the functional relationship between the ATR-DDR and mTOR pathway function, I examined lines from DS patients and other mTOR conditions.

5.1.1 INSR and Donohue syndrome

The human insulin receptor (INSR) is formed of two α subunits (extracellular, that bind insulin) and two β subunits (with intracellular receptor tyrosine kinase (RTK) domain), both coded by a single gene *INSR* on chromosome 19.

Donohue syndrome (DS; OMIM 246200), Rabson-Mendenhall syndrome (OMIM 262190) and type A insulin resistance (OMIM 610549) are examples of insulin-resistant syndromes resulting of *INSR* mutations associated with differing levels of residual activity. The one with the most severe clinical presentation is DS, initially referred to as Leprechaunism (William Donohue, 1948), which represents also the first *INSR* mutation found in a human disorder. DS is a rare autosomal recessive condition (estimated 1 in 4 million live births (Taylor et al. 1992), characterized by severe intrauterine and post-natal growth retardation, characteristic small “elfin-like” facies (Figure 5.1.3), insulin-resistant diabetes mellitus, *acanthosis*

nigricans, hyperinsulinemia, failure to thrive, and early death. See Table 5.1.1 for a list of DS clinical manifestations.

Table 5.1.1. Clinical manifestations associated with Donohue syndrome.

Clinical features associated with Donohue syndrome
Hypertrichosis
Pachyderma
Acanthosis nigricans
Prominent genitalia with hypertrophy
Mammary hyperplasia
“Elfin-like” facial features
<i>Prominent eyes with hypertelorism</i>
<i>Broad nose with wide nostrils</i>
<i>Thick lips</i>
<i>Large low set ears</i>
Hirsutism
Poor muscular development
Low birth weight
Reduced fat stores/ lipoatrophy
Neonatal growth retardation
Dysplastic nails
Hyperplasia and rugation
Joint laxity
Metabolic dysfunction
Endocrine dysfunction
Failure to thrive



Figure 5.1.3. Characteristic facial features and clinical presentation of Donohue syndrome patients.

Donohue's syndrome patients display dysmorphic facies, including large low set ears, saddle nose deformity, but also hirsutism and hypertrichosis, reduced subcutaneous fat, *acanthosis nigricans*, low birth weight. (Diabetes mellitus: a fundamental and clinical text, Derek leRoith et al. 3rd ed, 2004 by Lippincott Williams & Wilkins; Principles of Diabetes mellitus, Leonid Poretsky, 2002, Kluwer Academic Publishers)

Donohue syndrome patient-derived cells present with extremely reduced insulin binding capacity or receptor autophosphorylation/kinase activity (D'Ercole et al. 1979; Kobayashi et al. 1978). *INSR* knockout in the mouse results in only slight prenatal growth retardation, quickly developing postnatal diabetes followed by early death due to ketoacidosis (Kitamura et al. 2003). The differences found between the mouse model and humans lacking *INSR*, were attributed to distinct development timing and compensatory effect of Igf1 receptor signaling in mice (Kitamura et al. 2003).

5.1.2 PTEN and Cowden's disease

Cowden's disease (CD: OMIM 158350) is a rare autosomal dominant syndrome characterized by multiple hamartomas (Lloyd and Dennis 1963). The main features of the disease include macrocephaly, multiple hamartomatous lesions in various tissues (such as skin, gastrointestinal tract, brain, endometrium, breast), mild to moderate mental retardation, and developmental delay. Importantly, CD is associated with increased risk of developing breast, thyroid, and endometrial cancers.

Cowden's disease results of germline mutations in *PTEN* (Phosphatase and tensin homolog) a phosphatidylinositol-3,4,5-trisphosphate (PIP₃) phosphatase that negatively regulates PIP₃ intracellular levels. PTEN functions as a tumor suppressor by negatively regulating PI3K/AKT signaling. *PTEN* germline mutations are found in a group of disorders known as the PTEN hamartomatous tumor syndromes (PHTS), which also include the Lhermite-Duclos disease (OMIM 158350), Bannayan-Riley-Ruvalcaba syndrome (OMIM 153480) and Proteus syndrome (OMIM 176920) (Liaw et al. 1997). *PTEN* null mice die during embryogenesis. Mice heterozygous for PTEN develop tumors in several tissues, including breast, colon and endometrium (Stambolic et al. 2000).

PTEN has been implicated in regulation of genomic stability. PTEN upregulates the DSB repair protein, RAD51 (Shen et al. 2007); and is involved in modulation of the G2/M checkpoint, whereby PTEN loss can result in AKT-mediated inhibition of CHK1 function (Puc et al. 2005; Puc and Parsons 2005).

ATR-function defective cells, such as Seckel syndrome, are compromised in insulin/AKT/mTOR signaling (Chapter 4). In this chapter I set out to investigate if, conversely, specific primary defects in mTOR signaling impact on the DNA damage response, and If so by what/which mechanism(s), and what could be the consequences at the human syndromal level.

5.2 Results

5.2.1 Donohue's syndrome and Cowden's disease patient cells exhibit defective ATR-dependent G2/M checkpoint arrest

To determine whether primary defects in the insulin/PI3K/mTOR axis could manifest with DNA damage response defects, thereby phenocopying ATR-defective Seckel syndrome, I analyzed INSR-defective Donohue syndrome (GM16378, CAO133) and PTEN-defective Cowden disease (GM10080) cell lines for their ability to respond to DNA damage caused by UV and IR.

The G2/M checkpoint prevents cells from entering mitosis harboring DNA damage, and can be activated by either ATM or ATR, depending on the type of DNA damage incurred (Sancar et al. 2004) (see Introduction). To analyze the early G2/M checkpoint, the cells were irradiated with either 7J UV or 3Gy IR, incubated with the microtubule-depolymerizing drug, colcemid and the percentage of mitotic cells assessed after 4 hours. In control (WT) cells, the mitotic cell population significantly decreases when exposed to UV radiation. By contrast, ATR-defective Seckel syndrome cells (ATR-S) are defective in the activation of this checkpoint and a significant population of cells enters mitosis prematurely after UV treatment.

Surprisingly, both DS and CD patient-derived LBLs showed inefficient UV radiation-induced G2/M checkpoint arrest (Figure 5.2.1.) suggesting impairment of the ATR-regulated DNA damage response pathway. In contrast, all cell lines efficiently arrested after exposure to gamma radiation, which induces the ATM-dependent DDR. This suggests the checkpoint impairment observed in DS and CD cells to be specific to ATR (but not ATM)-dependent signaling. Furthermore, DS and CD patient-derived cells similarly failed to arrest proliferation up to 24hrs following UV irradiation (data not shown), when compared to control LBLs.

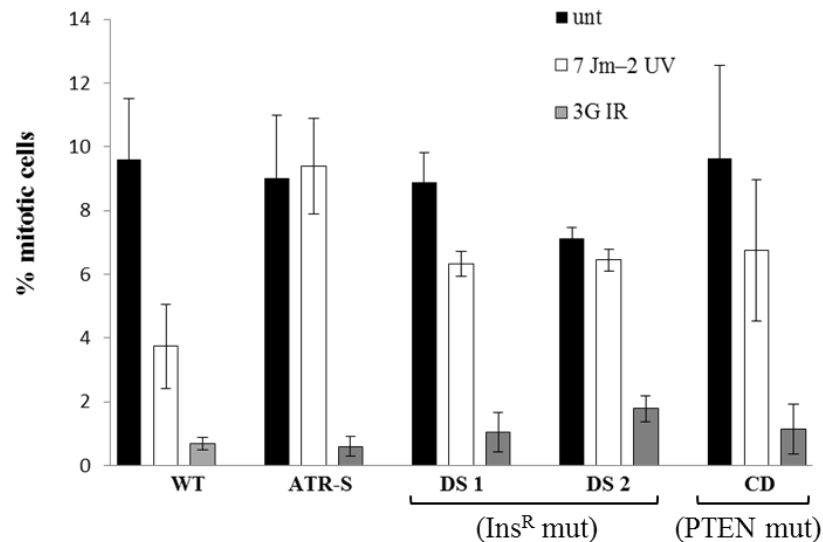


Figure 5.2.1. Donohue's syndrome and Cowden's disease patient cells exhibit defective ATR-dependent G2/M checkpoint arrest.

Control (WT=AG87), ATR-defective (ATR-S), Donohue syndrome (DS1=GM16378; DS2=CAO133) and Cowden disease (CD=GM10080) cells were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and the number of mitotic cells assessed 4hrs later. ATR-S, and both DS and CD patient derived cells failed to efficiently activate the ATR-dependent G2/M checkpoint arrest, compared to control cells. By contrast, the IR-induced (ATM dependent) checkpoint was efficiently activated under these conditions. Data represents the mean±SD from 3 separate experiments.

5.2.2 Insulin receptor knockdown results in defective ATR-dependent G2/M arrest

To confirm that the striking checkpoint phenotype observed in Donohue syndrome patient cells was due to Insulin receptor deficiency, I inhibited the *INSR* by siRNA in MG63 osteosarcoma cells (Figure 5.2.2a) and analyzed the checkpoint proficiency of these cells. siRNA against the *INSR* resulted in a less efficient induction of the G2/M checkpoint arrest in response to UV treatment, when compared to WT cells (Figure 5.2.2b). Significantly, this defect was again restricted to treatment with UV, but not IR. This result reinforces the checkpoint defect data on the Donohue syndrome patient cells, suggesting impairment of the ATR-dependent DNA damage response specifically in cells compromised in insulin receptor signaling.

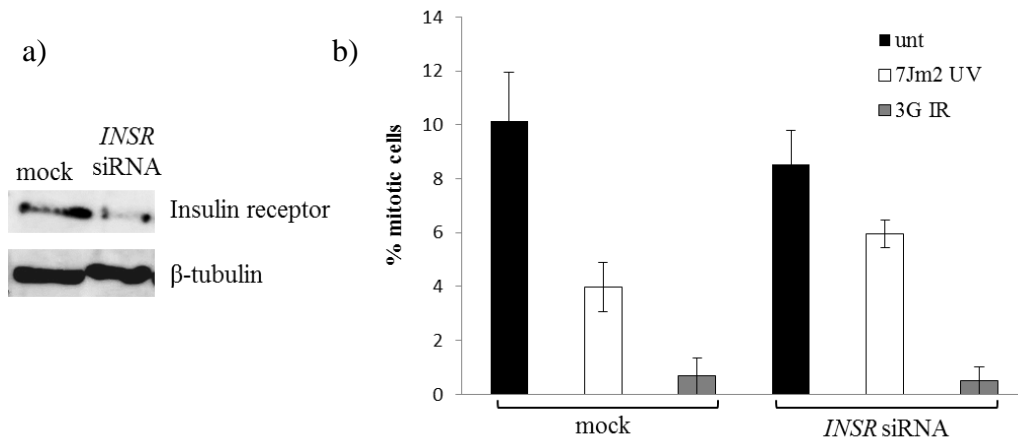


Figure 5.2.2. Insulin receptor knockdown results in defective ATR-dependent, but not ATM-dependent, G2/M DNA damage checkpoint.

a) siRNA mediated *INSIR* knockdown in MG63 osteosarcoma cells was confirmed by western blot. β -tubulin levels were used for protein loading. **b)** 48h after siRNA, the cells were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and number of mitotic cells assessed 4hrs later. After *INSIR* knockdown, the cells fail to activate the UV-induced G2/M checkpoint arrest as efficiently, when compared to control (mock). *INSIR* siRNA cells exhibit IR-induced checkpoint arrest. Data represents the mean \pm SD from 3 separate experiments.

5.2.3 ATR-dependent 53BP1 foci formation is less efficient in Donohue syndrome and Cowden disease LBLs.

Following DNA damage, 53BP1 is phosphorylated and rapidly localizes to nuclear foci. The precise role of 53BP1 in the DNA damage response remains unclear but it has been implicated in the regulation of G2/M checkpoint activation (B. Wang et al. 2002). Inhibition of DNA replication by the ribonucleotide reductase inhibitor, hydroxyurea (HU) also induces 53BP1 foci formation, specifically in an ATR and CHK1-dependent fashion, at the arrested replication fork in S phase cells (Sengupta et al. 2004; Stiff et al. 2006).

I looked at the formation of HU-induced 53BP1 foci in Cowden and Donohue syndrome cells, one of the earlier observed DNA damage response events (Anderson et al. 2001). Both DS and CD cells formed lower amounts of HU-induced 53BP1 foci (Figure 5.2.3) when compared to control cells (WT), although the level of induction was similar to WT cells, particularly for the CD cells.

This data suggests that aspects of the ATR-dependent DDR may be less efficient in DS and CD cells. PTEN deficiency has previously been linked to CHK1

dysfunction (Puc et al. 2005; Puc and Parsons 2005), therefore I decided to concentrate on further characterizing the novel ATR-pathway defect in Donohue syndrome.

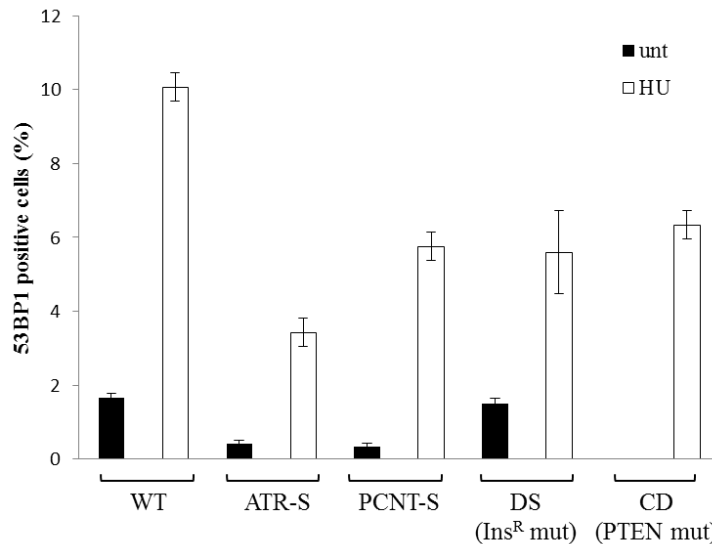


Figure 5.2.3. ATR-dependent 53BP1 foci formation is impaired in Donohue syndrome and Cowden disease LBLs.

Donohue syndrome (DS) and Cowden disease (CD) LBLs are less efficient in forming 53BP1 foci, when compared to control (WT) cells. All LBLs were treated with 5mM HU for 2 hrs. Positive cells were scored as those with more than 5 foci, as determined by immunofluorescence against 53BP1. Data represents the mean \pm SD from 3 separate experiments.

5.2.4 Activation of ATR-dependent substrates in Donohue syndrome cells after treatment with DNA damaging agents.

The G2/M cell cycle arrest is strongly dependent on CHK1-mediated signaling, which leads to suppression of CDK1-CyclinB1 activity via CDC25A. CDC25A phosphatase is a positive regulator of cell cycle transitions, specifically at the G2/M border where it removes the inhibitory T14 and Y15 phosphorylation on CDK1 to allow activation of CyclinB-CDK1 complex which controls progression from G2 into M (Nyberg et al. 2002; Sancar et al. 2004) (Figure 5.2.4).

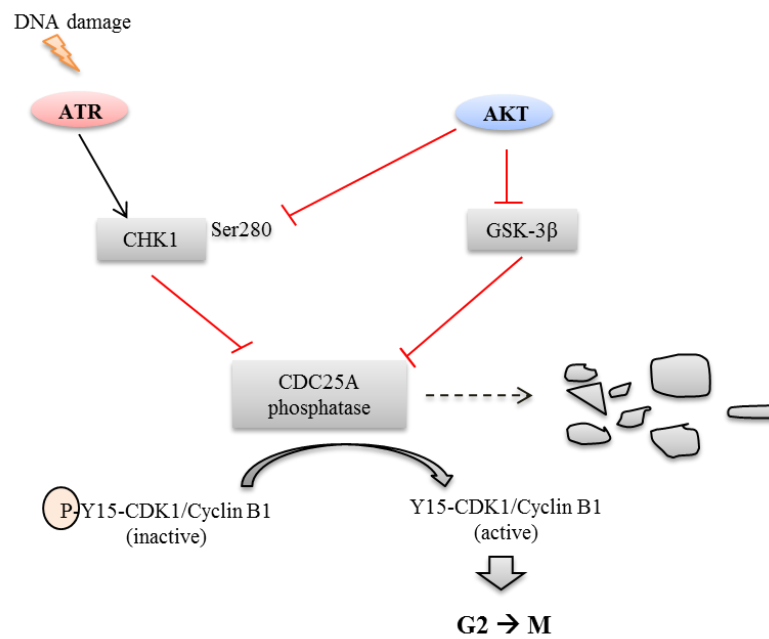


Figure 5.2.4. Model for CDC25A regulation involving the DNA damage response pathway and the AKT pathway.

Checkpoint-deficient ATR-Seckel syndrome cells fail to activate CHK1 via phosphorylation at Ser345 following HU treatment. DS cells however, showed proficient HU-induced phosphorylation of CHK1 on Ser345 (Figure 5.2.5a). This observation suggests that the checkpoint impairment observed in these cells likely lies downstream of CHK1 activation by ATR.

CHK1 is also directly phosphorylated at Ser280 by AKT, a modification that is associated with increased CHK1 cytoplasmic localization, and is thought to inhibit checkpoint activation (King et al. 2004; Puc and Parsons 2005; Shtivelman et al. 2002). CHK1 phosphorylation at Ser280 in response to HU was also normal in DS cells (Figure 5.2.5.a), compared to WT cells. Interestingly, ATR-S cells failed to induce Ser289 CHK1 phosphorylation under the same conditions.

In checkpoint proficient cells (WT), UV-induced DNA damage results in inactivation and downregulation of CDC25A levels via β TrCP mediated ubiquitination, via a CHK1-dependent process delaying mitotic entry (Figure 5.2.5b) (Deng 2009; Busino et al. 2003; Ahel et al. 2009). Significantly, CDC25A inhibition is less sustained in DS patient cells. CDC25A overexpression has been reported in a number of cancers where it is associated with poor prognosis (Harvard et al. 2011; Kristjánsdóttir et al. 2004).

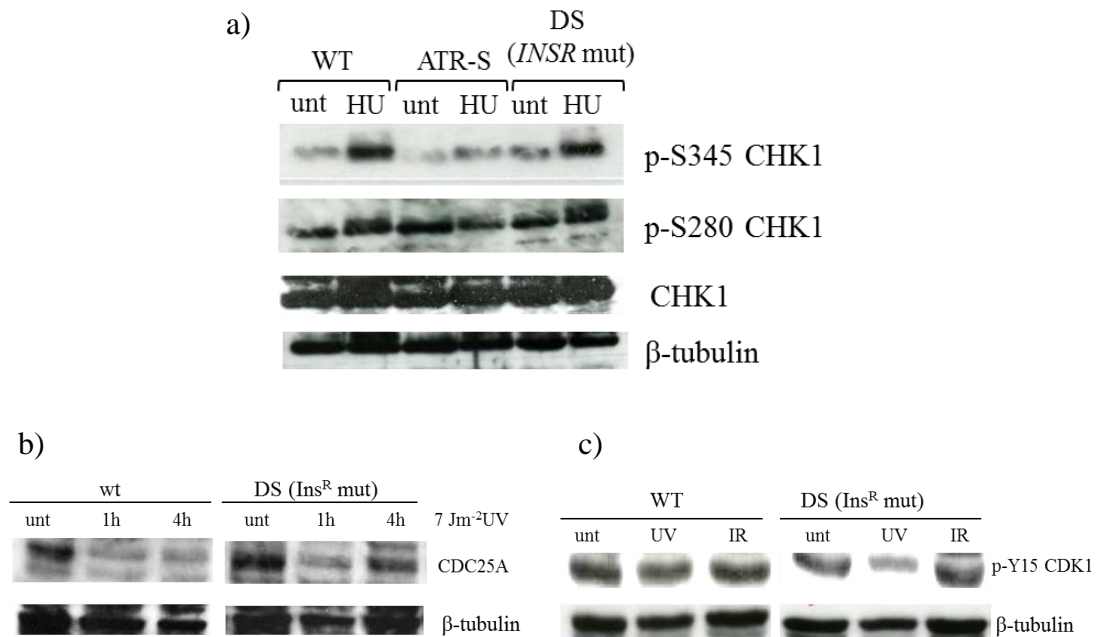


Figure 5.2.5. Activation of ATR-dependent substrates in Donohue syndrome cells after treatment with DNA damaging agents.

a) Phosphorylation of CHK1 at S345 and S280 after 5mM HU, 2hrs, is not defective in Donohue syndrome (DS) patients cells, similar to control (WT) cells and by contrast to ATR-S cells. CHK1 levels are not reduced in DS cells. **b)** DS (GM16378) LBLs show increased levels of CDC25A phosphatase 4h after 7Jm⁻²UV, when compared to WT cells. **c)** Correspondingly, DS cells exhibited reduced levels of p-Y15 CDK1 after UV (but not IR), when compared to control (WT). WT and DS LBLs were irradiated with 10Jm⁻² UV or 3Gy ionizing radiation, incubated for 4h and analyzed by Western-blot using specific antibodies. β-tubulin levels were used as control for protein loading.

Correspondingly, the inhibitory phosphorylation of CDK1 was reduced in DS cells, at 4h after UV (but not IR) treatment, when compared to WT (Figure 5.2.5c). This suggests an impact specifically on the ATR-dependent checkpoint. Inefficient CDC25A inhibition, and consequently increased activation of the CDK1/CyclinB complexes in Donohue syndrome would lead the cells prematurely into mitosis.

5.2.4 Increased phosphorylation of AKT and GSK-3 β in response to UV in DS cells

Due to the known role of AKT in the checkpoint response, I looked at AKT activation in *INSR*-defective DS cells after UV-induced DNA damage. AKT is activated by phosphorylation on Thr308 and Ser473. AKT is phosphorylated on Thr308 by PDK1, downstream of insulin/PI3K signaling.

I found increased basal level of activated Thr308 AKT in DS cells, compared to control cells (Figure 5.2.6) likely a consequence of compensatory signaling via PDK1/PDK2 due to the *InsR* defect. Studies have shown that AKT activity is elevated in the G2/M phase (B. B. Kahn et al. 2005; Shtivelman, Sussman, and Stokoe 2002) and activation of AKT has been shown to overcome G2 arrest in response to ionizing radiation (Young 2009; Kandel et al. 2002) and other forms of DNA damage inducing agents (B. B. Kahn et al. 2005; Hirose et al. 2005). This suggests that AKT signaling is able to influence the outcome of this checkpoint regardless of which checkpoint signaling pathway is being activated. The *level* of AKT activation is likely to have a role in how AKT can affect the regulation of these checkpoint responses. The activity of a number of proteins involved in cell cycle regulation can be modulated by AKT, such as CHK1 (S280) and TopBP1 (S1159) (Garelick and Kennedy 2011; N. Xu et al. 2012).

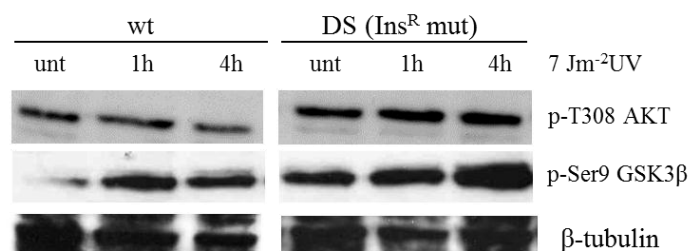


Figure 5.2.6. UV-induced AKT and GSK3 β phosphorylation in *INSR*-defective DS cells.

Basal levels of phosphorylated AKT at Thr308 are elevated in Donoué syndrome (DS) patient-derived cells when compared to WT cells. p-Thr308 AKT levels are maintained after UV irradiation, for the conditions tested (7Jm⁻²UV, up to 4hrs). GSK3 β phosphorylation (and *inactivation*) increases in response to UV treatment in both WT and DS cells. Additionally, levels are higher in DS LBLs show increased levels of p-Ser9 GSK3 β , when compared to WT cells, both at the basal level as well as after UV induction. β -tubulin levels were used as control for protein loading.

However, my data suggests that the putative site of action of AKT in mediating the G2/M checkpoint defect in DS cells is likely to act downstream or parallel to CHK1 activation (Figure 5.2.4).

An interesting recently found link between insulin signaling and cell cycle/checkpoint regulation involves the glycogen synthase kinase-3 β (GSK-3 β), which is directly phosphorylated by AKT on Ser9, resulting in GSK-3 β *inhibition*. GSK-3 β directly phosphorylates CDC25A on S76 and targets it for proteolysis (Young 2009; Kang et al. 2008) (Figure 5.2.6b).

I found GSK-3 β is phosphorylated, and thus *inactivated*, in response to DNA damage (10J UV) in normal cells. Remarkably, GSK-3 β pS9 levels were further elevated in Donohue Syndrome cells when compared to WT levels (Figure 5.2.6). This result suggests that GSK-3 β *inhibition* may affect the total levels of CDC25A present in these cells, independently of CHK1 activity, and thus influence the checkpoint response at the G2/M transition.

5.2.6 GSK-3 β inhibition results in defective ATR-dependent G2/M arrest.

To further characterize the potential effect of GSK-3 β on the ATR-dependent checkpoint defect observed in Donohue syndrome, I introduced a siRNA-mediated knockdown of *GSK-3 β* in MG63 osteosarcoma cells (Figure 5.2.7a). *GSK-3 β* knockdown cells maintained a high mitotic index in response to DNA damage caused by UV irradiation, but efficiently arrested following gamma irradiation (Figure 5.2.7b). This data suggests that GSK-3 β is a regulator of the ATR-dependent G2/M checkpoint specifically, suggesting a mechanism for the checkpoint defect observed in DS patient cells.

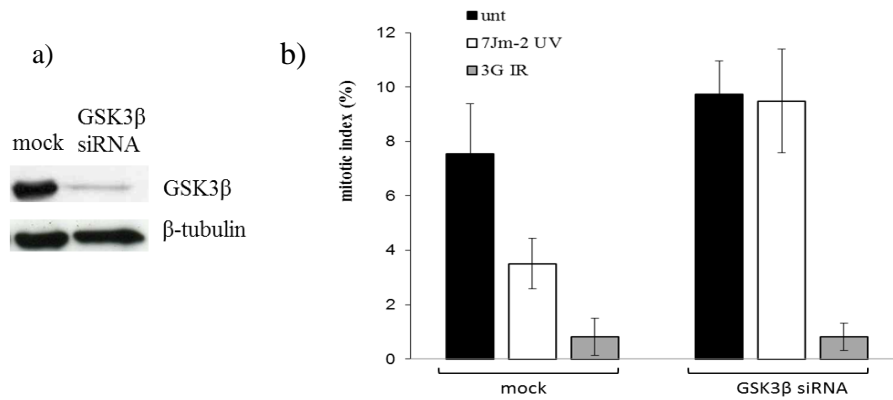


Figure 5.2.7. GSK-3 β knockdown results in defective ATR-dependent, but not ATM-dependent, G2/M DNA damage checkpoint.

a) siRNA mediated *GSK-3 β* knockdown in MG63 osteosarcoma cells was confirmed by western blot. β -tubulin levels were used for protein loading. **b)** 48h after siRNA, these cells were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and number of mitotic cells assessed 4hrs later. After *GSK-3 β* knockdown, the cells fail to activate the UV-induced G2/M checkpoint arrest as efficiently, when compared to control (mock). *GSK-3 β* siRNA cells exhibit IR-induced checkpoint arrest. Data represents the mean \pm SD from 3 separate experiments.

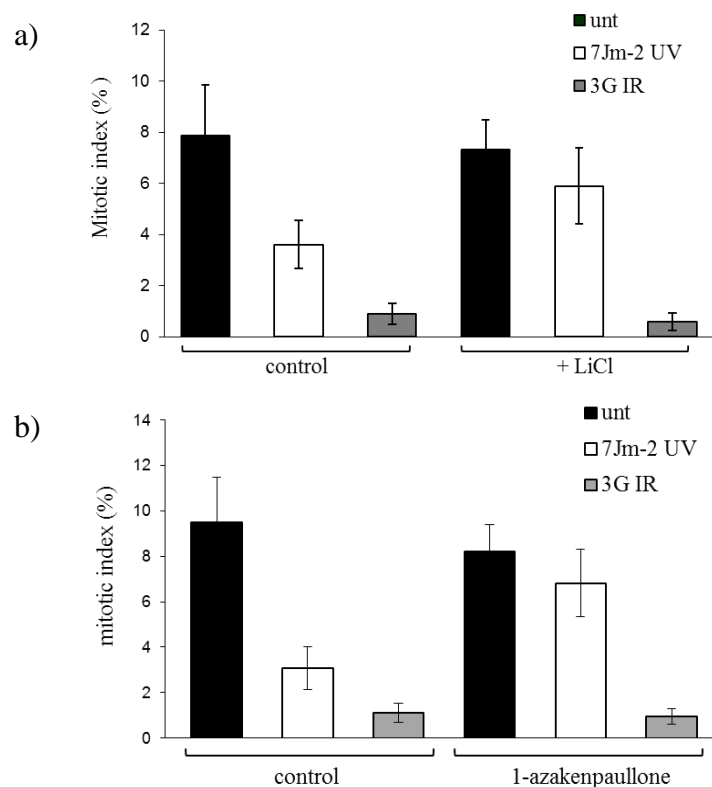


Figure 5.2.8. GSK-3 β inhibition results in defective ATR-dependent, but not ATM-dependent, G2/M DNA damage checkpoint

Treatment with GSK-3 β inhibitors **a)** lithium chloride (LiCL), and **b)** 1-AZKP results in failure to activate the G2/M checkpoint in response to UV irradiation. The WT LBLs were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and number of mitotic cells assessed 4hrs later. GSK-3 β inhibition does not compromise the IR-induced checkpoint arrest. Data represents the mean \pm SD from 3 separate experiments.

Moreover, this data was also supported by the use of GSK-3 β kinase inhibitors. Lithium is a crude GSK-3 β inhibitor that has been extensively investigated for the treatment of neuropathologies and neurodegenerative disorders, such as schizophrenia and Alzheimer's disease, which are associated with increased activation/over-expression of GSK-3 β . Lithium is thought to compete directly with magnesium at the cationic binding site in GSK-3 β , inhibiting its catalytic activity. Reports suggest also a potential indirect inhibitory effect of lithium over GSK-3 β by activating AKT (Young 2009; Chalecka-Franaszek et al. 1999; Zhang et al. 2003) or by reducing GSK-3 β mRNA levels (Young 2009; Mendes et al. 2009). I found lithium treatment of WT LBLs resulted in failure to arrest cell proliferation in response to UV, though the cells remained proficient in activating the checkpoint response to DNA double strand breaks caused by IR. 1-azakenpaullone (1-AKP) is among the most potent and selective small molecule GSK-3 β inhibitors that have since been developed. Treatment with 1-AKP also resulted in defective ATR-dependent G2/M checkpoint (Figure 5.2.8). Collectively, this data indicates that the kinase activity of GSK-3 β is required for the regulation of this checkpoint.

5.2.7 UV induced G2/M checkpoint after AKT inhibition

Several upstream kinases other than AKT are able to inhibit GSK3 β by phosphorylation on Ser9. These include protein kinases A (PKA) and C (PKC), p90 ribosomal S6 kinase (p90RSK) and p70 ribosomal S6 kinase (p70S6K) (Fang et al. 2000; Kim et al. 2007; Stambolic et al. 1994, Sutherland et al. 1993).

I used an AKT inhibitor to determine whether the checkpoint defect observed in DS is AKT-dependent. AKT inhibition by treatment with 1.5 μ M of AKT inhibitor IV (InSolution™ AKT Inhibitor IV, cat.124015, Calbiochem) was confirmed by looking downstream at GSK3 β phosphorylation by western blotting (Figure 5.2.9a). Some residual GSK3 β inhibition was still observed in DS cells, as well as PCNT-S cells after treatment with the inhibitor, upon UV irradiation. In chapter 4 I showed that PCNT-deficient cells are compromised in the PI3K-dependent response to insulin, and the data also suggested increased activation of AKT in those cells.

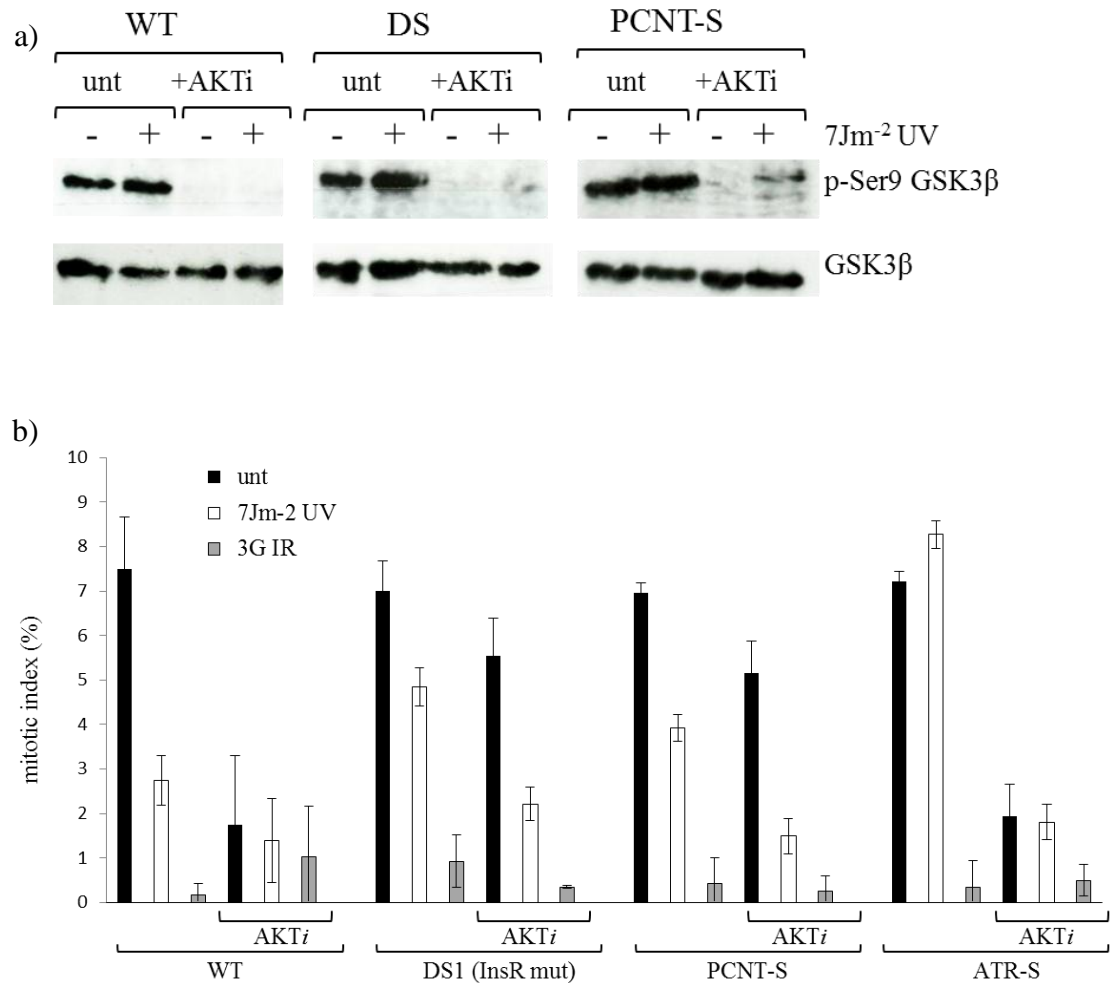


Figure 5.2.9 AKT inhibition rescues the ATR-dependent G2/M DNA damage checkpoint defect in Donohue syndrome.

a) AKT inhibition on WT, DS, PCNT-S LBLs using 1.5 μ M of AKT inhibitor (InSolution™ AKT Inhibitor IV, cat.124015, Calbiochem) results in decreased phosphorylation of GSK3 β on Serine 9, confirmed by western blot. Total GSK-3 β levels were used for loading control. **b)** Treatment with AKT inhibitor results in efficient G2/M arrest after UV treatment in Donohue syndrome patient cells, thereby rescuing the checkpoint defect in these cells. Under these conditions, AKT inhibition resulted in decreased cell proliferation in both WT, as well as ATR-S, even in un-irradiated cells. Interestingly, the use of AKT inhibitor also rescued the G2/M checkpoint defect in PCNT-deficient cells (PCNT-S). The cells were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and number of mitotic cells assessed 4hrs later. AKT inhibition did not impact the IR-induced checkpoint arrest, under these conditions. Data represents the mean \pm SD from 3 separate experiments.

At this concentration, AKT inhibitor IV is known to block cell proliferation, and indeed control (WT) LBLs showed a drastically reduced mitotic index upon AKT inhibitor IV treatment (Figure 5.2.9.b). However, DS cells are resistant to inhibition of proliferation by AKT IV under the same conditions, which supports the observation that AKT is hyperactive in these cells. Strikingly, treatment with AKT inhibitor rescues the G2/M checkpoint defect in DS cells, which indicates that AKT plays a role in the modulation of this checkpoint.

Interestingly, AKT inhibition also rescued the ATR-dependent G2/M checkpoint defect in PCNT-S, but not in ATR-S patient derived cells suggesting that different mechanisms are behind the DDR defect in these cells. This observation is consistent with a defect in PCNT-S cells affecting a more upstream point in the insulin-PI3K-AKT cascade, likely the PI3-kinase itself. Conversely, the defect in ATR-S would impact on the insulin pathway downstream of AKT activation, and therefore not benefit from AKT inhibition.

AKT inhibition did not affect other aspects of the DDR. 53BP1 foci formation was not altered by treatment with the AKT inhibitor, both in DS as well as PCNT-S cells (Figure 5.2.10) further suggesting that this influence is downstream of CHK1 activation.

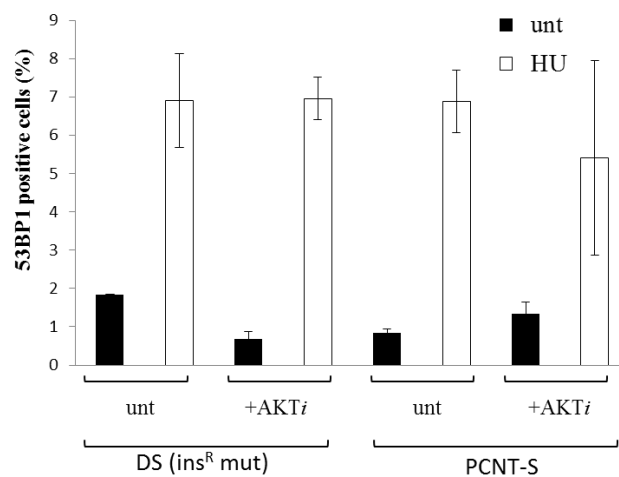


Figure 5.2.10. AKT inhibition does not affect 53BP1 foci formation in Donohue syndrome.

a) AKT inhibition using 1.5 μ M of AKT inhibitor (InSolution™ AKT Inhibitor IV, Calbiochem) does not increase the number of 53BP1 foci formed in response to HU-treatment, to levels comparable to WT cells. the same is also true for PCNT-S cells. Donohue syndrome (DS) and PCNT-defective MPDII (PCNT-S) LBLs were treated with 5mM HU for 2 hrs. Positive cells were scored as those with more than 5 foci, as determined by immunofluorescence against 53BP1. Data represents the mean \pm SD from 3 separate experiments.

5.3 Summary

The overlap/interplay between the DDR and the insulin-AKT-mTOR pathway has been previously described. In the previous chapter I described defects in insulin/mTOR pathway signaling in cells derived from ATR and PCNT defective Seckel syndrome patients, both manifesting defects in the ATR response. Severe microcephaly and growth retardation are clinical features of SS, which are also observed in Donohue syndrome, caused by a mutation in the insulin receptor. In this chapter I set out to investigate if primary defects in a metabolic disorder, harboring mutation in the insulin/mTOR pathway, such as Donohue syndrome, could also impact on the DNA damage response.

I found *INSR*-defective DS cells (and *PTEN*-defective Cowden's disease cells) exhibit defective ATR-dependent G2/M activation. This defect was also observed following siRNA-mediated *INSR* knockdown. Activation of ATR-dependent substrates was also evaluated, and DS cells exhibited elevated levels of CDC25A, and reduced phosphorylation of Y15-CDK1, in response to UV irradiation specifically. Additionally, increased phosphorylation of AKT and GSK-3 β was also observed in DS cells under the same conditions. Surprisingly, the ATR-dependent G2/M checkpoint defect in DS was rescued by GSK-3 β inhibition, mediated by GSK-3 β siRNA or by use of specific GSK-3 β kinase inhibitors. Furthermore, AKT inhibition also rescued the checkpoint defect in DS (and PCNT-S cells). This data suggests that both GSK-3 β and AKT play a role in modulation of this checkpoint.

5.4 Discussion

Patient-derived cell lines from two distinct human disorders caused by primary defects in mTOR pathway, Donohue syndrome and Cowden's disease, have been examined for the ability to effect aspects of the DNA damage response. These disorders, particularly DS, display a set of clinical features that in some aspects relates to Seckel syndrome/MOPD-type II. Both INSR-defective DS cells and PTEN-defective CD cells show defective UV-induced G2/M checkpoint activation. Both cell lines efficiently activated the checkpoint in response to IR, suggesting a problem specific in the ATR-regulated branch of the DNA damage response. A role for PTEN in checkpoint regulation has previously been established. Interestingly, PTEN null ES cells were shown to have a deficient IR-induced G2/M arrest (Kandel, 2002). I present here the first association of an ATR-dependent checkpoint defect in a human disorder caused by PTEN deficiency. Loss of checkpoint proficiency could be a source of genomic instability in these cells, with implications for the high cancer predisposition observed in CD patients.

The association of an INSR-defect with a DNA damage response defect is novel. A knockdown of *INSR* in an independent cell line confirmed the checkpoint defect identified in the two Donohue syndrome patient cell lines.

In order to identify a possible mechanism behind this checkpoint defect, other aspects of the signaling response downstream of ATR were examined. Both DS and CD cells showed reduced, but not altogether defective, HU-induced formation of 53BP1 foci. Phosphorylation of S345-CHK1 after treatment with HU was normal in DS and CD cells, an ATR-dependent event. This contradicts previous observations of diminished CHK1 S345 phosphorylation in PTEN^{-/-} cells, associated with elevated p-S473 AKT (Xu et al. 2010). An AKT-mediated regulation of CHK1 function has been suggested, involving direct phosphorylation of CHK1 on S280 by AKT. Phosphorylated S280-CHK1 levels in DS, in response to HU, were also comparable to WT levels. In response to UV-induced DNA damage, CHK1 mediates the downregulation of CDC25A phosphatase to delay entry into mitosis. Importantly, CDC25A is not efficiently inhibited in Donohue syndrome patient cells in response to UV irradiation. This results in sustained de-phosphorylation of CDK1, meaning activation of the CDK1/Cyclin B complexes inappropriately, which drive the cell cycle into mitosis, and can account for the leaky checkpoint observed in these cells.

AKT is activated by phosphorylation on T308, mediated by the PDK1 kinase, in response to insulin/PI3K signaling. Impaired AKT activation is known to override cell cycle checkpoints and promote cell proliferation in the presence of DNA damage.

Significantly, levels of p-T308 AKT are higher in DS cells when compared to control cells, even prior to DNA damage. This could be related to lack of INSR-mediated feedback inhibition of this pathway and/or compensatory phosphorylation of PDK1 from PI3K. AKT activation has been shown to bypass the ATM-dependent G2/M checkpoint following gamma irradiation (Kandel et al. 2002). Donohue syndrome patient cells however, efficiently activate this checkpoint when treated with IR. It is possible that the *level* of basal AKT activation is important in modulating this response, and that the levels of activated AKT in Donohue syndrome are not high enough to surpass the ATM-dependent DNA damage response signal, which is activated in response to extensive double strand break –inducing DNA damage.

Because CHK1 phosphorylation is normal in DS, the putative target for an AKT-mediated checkpoint defect should lie downstream or parallel to CHK1 activation by ATR. The glycogen synthase kinase 3 β (GSK-3 β) is phosphorylated directly by AKT on serine9, resulting in its inhibition. Notably, GSK-3 β can directly phosphorylate CDC25A and target the phosphatase for degradation (Kim et al. 2002; Kang et al. 2008). Levels of CDC25 phosphatases are carefully regulated during the cell cycle. CHK1 and GSK-3 β would then cooperate to regulate CDC25A proteolysis.

I found GSK-3 β inhibitory phosphorylation on serine 9 is induced by treatment with UV. Notably, GSK-3 β inhibition is more pronounced in DS when compared to control cells. By introducing a GSK-3 β knockdown in an independent cells system, and treating the cells with specific GSK-3 β inhibitors, I confirmed that GSK-3 β is a regulator of this checkpoint. Moreover, in DS cells this effect is dependent of AKT, as treatment with a specific AKT inhibitor rescues the checkpoint defect, in both DS but also in PCNT-S cells, and unlike the ATR-S cells. This data further suggests that different mechanisms underlie the DDR defect in ATR and PCNT-defective SS/MOPDII cells. Furthermore, this is consistent with a defect in PCNT-S cells affecting a more upstream point in the insulin-PI3K-AKT cascade, likely the PI3-kinase itself, as suggested in chapter 4.

Other mechanisms cannot be ruled out, that could potentially affect the checkpoint defect observed. For example, activated AKT is also able to phosphorylate and inhibit the nuclear kinase Wee1A, on Ser642 (Katayama, 2005). Wee1A also catalyzes the inhibitory phosphorylation on Y15-CDK1, resulting in activation of the CDK1/Cyclin complex. These findings contribute to understanding the mechanisms by which AKT modulates DNA damage signaling, which could have broader implications, since impaired checkpoint proficiency can be a cause for genomic instability in cells with elevated AKT activity. AKT misregulation is a known cause of enhanced proliferation and survival in tumor cells.

According to this model, impaired regulation of the insulin/AKT pathway, such as seen in DS, can result in deregulation of GSK-3 β activity, thus affecting the net balance of CDC25A regulatory events. Sustained levels of CDC25A in these cells would enforce the continued activation of the CDK-Cyclin complexes, leading the cells through the G2/M border in an untimely manner (Figure 5.3.1).

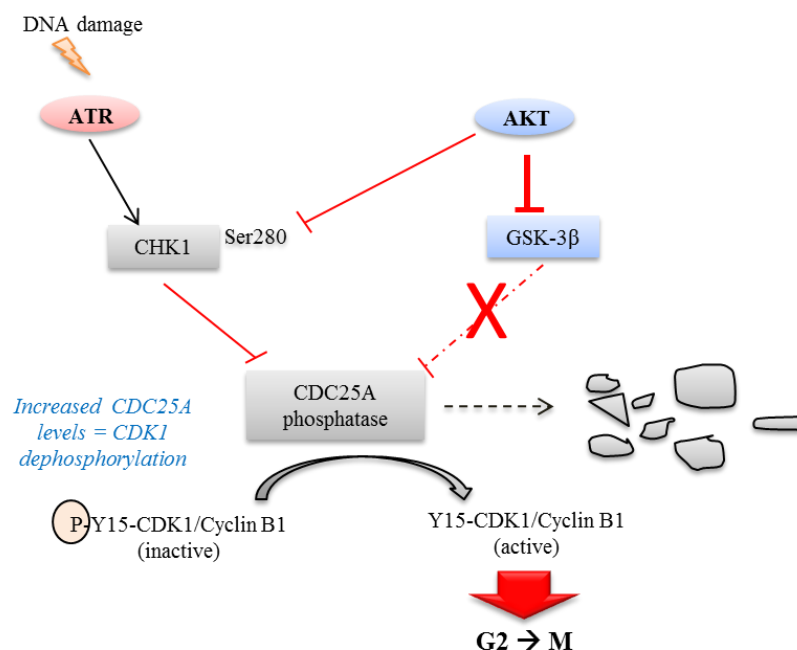


Figure 5.3.1. Model for G2/M checkpoint deficiency in Donohue syndrome.

However, other not necessarily mutually exclusive mechanisms may be at play that account for the clinical presentation of Donohue syndrome. One study showed downregulation of genes involved in cell growth and differentiation in patients with DS (Leick et al. 2010; Melis et al. 2003).

The Wnt inhibitor, Dickkopf 1 (DKK1) is overexpressed in DS patients, which also have compromised Wnt signaling, important during development. Kahn et al. 2005; Metcalfe et al. 2011) Binding of Wnt leads to GSK-3 β inactivation (Park et al. 2010).



The data presented in this chapter proposes that the AKT/GSK-3 β signaling pathway mediates an enhancement of the ATR/CHK1-dependent activation of the G2/M cell cycle checkpoint. This effect likely contributes to the specific checkpoint defect identified in two human disorders harboring mutations in the PI3K/AKT pathway, specifically Donohue syndrome. I identify novel routes to the manipulation of this cell cycle checkpoint in WT cells (GSK-3 β *i*) and even in patient-derived cells (AKT*i*). Whether the latter would be of benefit in the management of these conditions is worthy of further investigation. Investigation of the links between defects in insulin/mTOR pathway function with deregulation of the DNA damage response, should contribute to our increased understanding of the mechanisms and factors that modulate cell cycle checkpoints, as well as the clinical consequences of defects in these pathways and their particular manifestations in human disease.

Chapter Six

Results IV: Microcephaly - Capillary Malformation syndrome is caused by mutations in STAMBP resulting in aberrant RAS-MAPK and RTK-PI3K-AKT signaling

6.1 Introduction

In this chapter I will characterize molecular aspects of a newly reported microcephalic disorder that combines a number of features, such as severe microcephaly and growth delay that overlap with Seckel syndrome/MOPDII.

The Microcephaly-Capillary Malformation (MIC-CAP; OMIM 614261) Syndrome was first described in 2011. MIC-CAP patients exhibit severe congenital microcephaly (head circumference 3SD or more below mean) with progressive cortical atrophy, intractable epilepsy and multiple small capillary malformations (CMs) on the skin (Carter et al. 2011) (Mirzaa et al. 2011) (Isidor et al. 2011). Other clinical features include profound developmental delay, facial dysmorphism, small stature, spastic quadriparesis, and hypoplastic distal phalanges (Table 6.1.1; Figure 6.1.1).

One outstanding feature of all MIC-CAP patients is the presence, at birth, of numerous generalized capillary malformations on the skin also known as “port-wine stains” (Figure 6.1.1 F, G, H). These are cutaneous vascular abnormalities frequently observed in a class of developmental disorders associated with dysregulation of the RAS-MAPK (RAS-mitogen activated protein kinase) pathway, collectively referred to as *RASopathies* (Eerola et al. 2003). The RAS-MAPK signal transduction pathway is an important regulator of vascularization, cell growth and proliferation. The phenotypic features characteristic of *RASopathies* include cardiovascular anomalies, facial dysmorphism, skeletal and cutaneous abnormalities, neurocognitive delays and cancer, as exemplified by Noonan, LEOPARD (*l*entigines, *E*CG conduction abnormalities, *o*cular hypertelorism, *p*ulmonic stenosis, *a*bsnormal genitalia, *r*etardation of growth, *s*ensorineural *d*eafness), and Costello syndromes (Tidyman and Rauen 2009).

A defect in *RASA1* is known to cause a congenital capillary disorder called Capillary Malformation-Arteriovenous Malformation (CM-AVM; OMIM 608354) (Eerola et al. 2003) (Boon et al. 2005). The capillary lesions seen in CM-AVM patients are similar to those seen in MIC-CAP. The *RASA-1* gene codes for a protein with Ras-GAP (GTPase activating protein) activity, a negative regulator of the RAS-MAPK pathway (Ronnett et al. 2009; Bernardis et al. 2004). This defect results in

chronically active RAS signalling due to a failure to generate (recycle) GDP RAS from GTP RAS.

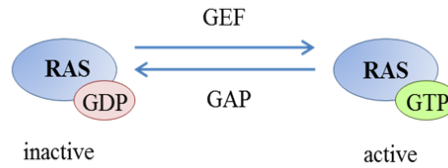


Table 6.1.1. Clinical features and molecular findings of MIC-CAP patients included in this study.

Patient	P1.2	P3.1	P5.1	P7.1
Exome Sequencing	+	+	+	–
Validated Mutations ^b (protein (cDNA))	p.Glu42Gly (c.125A>G) p.Arg178* (c.532C>T)	p.Phe100Tyr (c.299T>A) p.Arg424* (c.1270C>T)	nd	p.Arg38Cys (c.112C>T) nd
Gender	M	M	F	F
Age at assessment	9m	2y	5y4m	28m
Ethnicity	African-American	Caucasian	Caucasian	Caucasian
Gestational Age (weeks)	39	36	Term	37+2
Progressive congenital MIC	+	+	+	+
Small-for-gestational age	+	+	–	+
Birth OFC (SD)	-4	-2	-1.8	-8
Birth Weight (SD)	-1.5	-2	+1.8	-4
Birth Length (SD)	-2	nd	0 (mean)	-4
Later age	9m	17m	2y10m	28m
Later OFC - SD (age)	-6	-4	-2.5	-8
Later Weight (SD)	-4	-1 to -2	+2	-2.5
Later Length (SD)	nd	-2	0 (mean)	-4
Generalized CMs	+	+	+	+
Early-onset intractable seizures	+	+	+	+
Infantile spasms	–	–	–	nd
Hypoplastic distal phalanges	+	+	+	+
Global DD	+	+	+	+
Spastic quadriplegia	+	+	–	+
Myoclonus/clonus	–	+	–	+
Optic atrophy	+	+	nd	+
Simplified gyral pattern	+	+	nd	+
Increased extra-axial space	+	+	nd	+
Hippocampal hypoplasia	+	nd	nd	+
Hypomyelination	–	+	nd	+

This table summarizes the clinical findings in study participants, b. Numbering of mutations is relative to NM_006463 (gene) and NP_006454.1 (protein). Abbreviations: F: female; M: male; y: years; m: months; OFC: Occipito-frontal circumference; SD: values are shown by their s.d. value from the mean; ND: not determined; DD: developmental delay.

This condition establishes a biological precedent for hyperactive RAS signalling in a capillary abnormality (Turnley et al. 1999; Eerola et al. 2003). No *RASA1* mutations were found in MIC-CAP patients (Mirzaa et al., 2011).

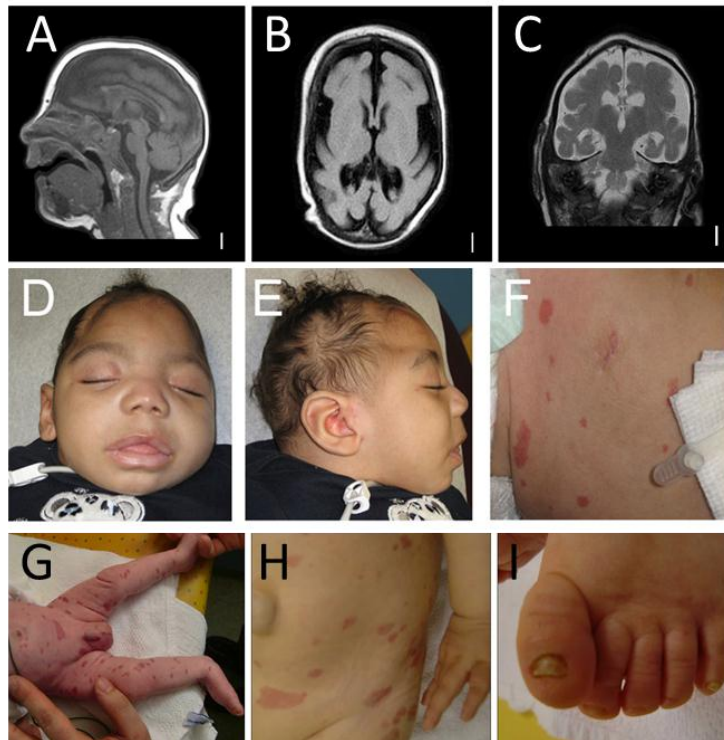


Figure 6.1.1. Neuroimaging and clinical features of MIC-CAP patients.

Images of the brain (A-C), evidencing the low-sloping forehead, simplified gyral pattern, increased extra-axial space, diffuse hypomyelination, and hippocampal hypoplasia. (D-F) Photos of MIC-CAP Patient 1.2 in this study (subject LR07-155a2, described in Mirzaa, 2011) at 9 months of age, showing the severe microcephaly, bitemporal narrowing with a low-sloping forehead and capillary malformations. Photos of MIC-CAP patient at 3 weeks (d) and 18 months (e) showing generalized capillary malformations of variable sizes and hypoplastic toenails.

6 MIC-CAP children, including 2 siblings, were reported in the initial clinical studies (Dasgupta and Milbrandt 2009; Carter et al. 2011; Mirzaa et al. 2011; Isidor et al. 2011). A total of 10 MIC-CAP patients were included in a study by our collaborators - Prof. W.B. Dobyns (Seattle, USA) and Dr. Kym Boycott (Ottawa, Canada) - who applied exome sequencing on 5 patients from unrelated families to identify the previously unknown MIC-CAP causative genetic defect(s). Novel recessive mutations were found in the gene *STAMBP* in MIC-CAP patients.

STAMBP (STAM binding protein, or AMSH - associated molecule with the SH3 domain of STAM) is an endosome-associated Zn(2+)-dependent ubiquitin isopeptidase that belongs to the JAMM metalloprotease family (Ronnett et al. 2009; Sierra, Wright, and Nash 2010). In addition to the JAMM (JAB1/MPN/MOV34) motif, the ubiquitously expressed STAMBP contains a microtubule-interacting and transport (MIT) domain and an SH3 binding motif (Figure 6.1.2) (Kahn et al. 2005; Tsang et al. 2006). STAMBP plays an important role in endocytosis, regulating endosomal sorting of ubiquitinated cargo (Qi et al. 2010; McCullough et al. 2006; Mattson et al. 2008; Agromayor et al. 2006; Kim et al. 2006; Mizuno et al. 2006; Kyuuma et al. 2007).

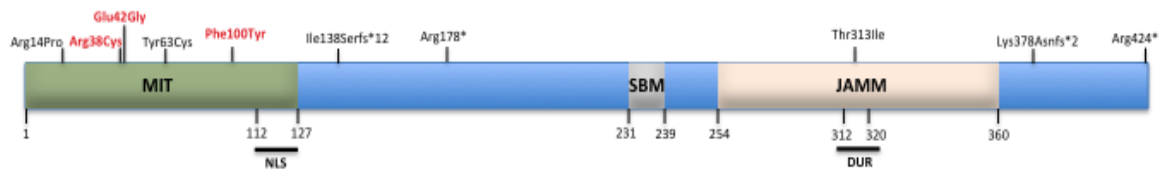


Figure 6.1.2 STAMBP protein structure showing the location of MIC-CAP causing mutations

The 424 amino acid sequence of STAMBP consists of the microtubule-interacting and transport (MIT) domain, the SH3 binding motif (SBM) (PX[V/I][D/N]RXXP), the JAMM (JAB1/MPN/MOV34) motif, the nuclear localization signal (NLS) and the distal ubiquitin recognition site (DUR). Five of the six missense mutations identified are located in the MIT domain, which is thought to be required for the interaction of STAMBP with CHMP3, an ESCRT-III subunit. In red are highlighted the mutations found in the MIC-CAP patient lines included in this study.

Sorting and trafficking of cell surface receptors is a highly regulated process important for maintenance of cell homeostasis and adequate response to extracellular stimuli, and enabling processes such as autophagy. STAMBP is recruited to the endosomal complexes required for transport (ESCRTs), a group of distinct macromolecules that mediates the sorting and trafficking of ubiquitinated proteins from endosomes to lysosomes (Dasgupta and Milbrandt 2009; Raiborg and Stenmark 2009). The ESCRTs DUBs include STAMBP and USP8, which interact with STAM (both associate with the SH3 domain of STAM via the same binding motif (PX[V/I][D/N]RXXKP) (Iascone et al. 2002; M. S. Kim et al. 2006). Together with Hrs, they form the ESCRT-0 complex, which recognizes and processes

ubiquitinated protein cargo at the early and sorting endosomes (O'Driscoll et al. 2003; Kyuuma et al. 2007; Wright et al. 2011). Endocytosed receptor-ligand complexes are either recycled back to the cell membrane or sorted to lysosomes for degradation in a process mediated by reversible ubiquitination (Figure 6.1.3) (Griffith et al. 2008; Komada 2008; Provot and Schipani 2005; Murga et al. 2009; Ma et al. 2007). This is a fundamental cellular process that controls the duration and amplitude of cell surface receptor-mediated signaling in a controlled, dynamic and reversible fashion (Matsuoka et al. 2007; Karsenty 2008; Raiborg and Stenmark 2009). STAMBP has also been reported to associate with CHMP proteins that interact with ESCRT-III (Ruzankina et al. 2007; Goldring et al. 2006; Tsang et al. 2006). These multiple interactions allow the ESCRT-associated DUBs to regulate multiple steps during endocytosis.

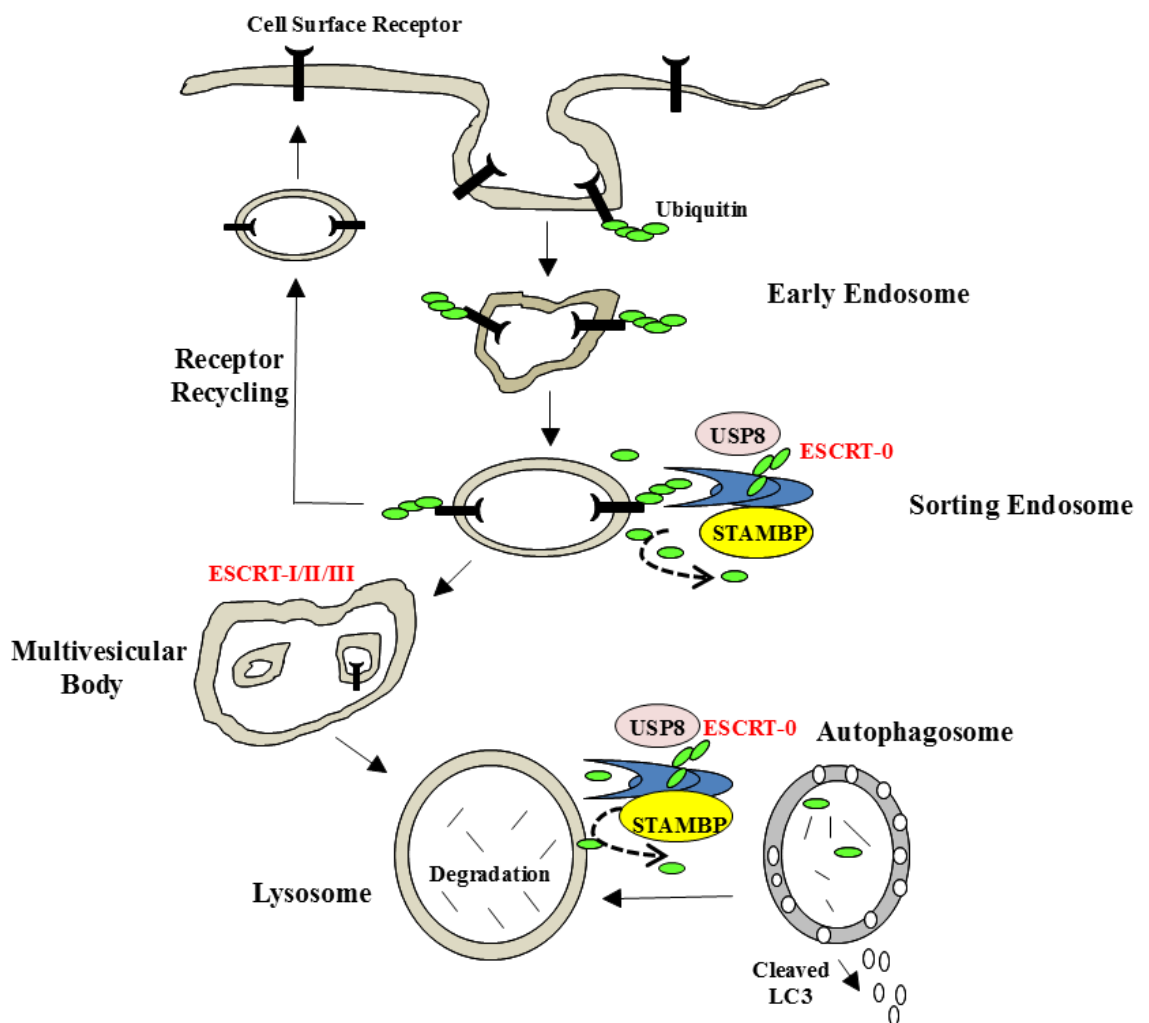


Figure 6.1.3 Model of STAMBP function in endocytosis.

STAMBP functions as an important DUB protease, along with USP8, that controls the sorting and trafficking of endocytosed, ubiquitinated receptor tyrosine kinases and G-protein coupled receptors.

Receptor ubiquitination is an important step in sorting and directing the receptor for recycling or for proteasome-mediated degradation. Depending upon the pathway, receptor mediated signaling can be either maintained or down-regulated. STAMBP interacts with early endosome sorting machinery via STAM and ESCRT complex machinery such as the ESCRT-III complex. ESCRT-DUBs are also thought to have a role in autophagy, a parallel degradation system that involves regulation through the ESCRT machinery.

Importantly, both ESCRT DUBs, STAMBP and UPS8 demonstrate distinct substrate preferences. STAMBP processes mainly K63-linked polyubiquitin (generally an activity modifying post-translational modifications), generating di-ubiquitin, on the other hand USP8 preferentially breaks-down K48-linked chains (linked to proteasomal degradation) to component ubiquitin monomers (Schipani 2006; Provot and Schipani 2007; McCullough, et al. 2004; Provot and Schipani 2005; McCullough et al. 2006). The DUB activity at the endosome therefore also recycles ubiquitin, and is important for the maintenance of free ubiquitin in the cells. Expression of an inactive STAMBP mutant results in accumulation of ubiquitin at the endosomes (Faqeih, et al. 2005; Sierra, et al. 2010; McCullough, et al. 2004).

Other pertinent STAMBP interactants include the Grb2 adaptor and the PI3K class II kinase (Figure 6.1.4) (Kitamura, et al. 2003; Sowa et al. 2009).

Interestingly, brain lesions containing ubiquitinated protein aggregates have been noted in *Stambp* knockout mice (Shah, et al. 2004; Suzuki et al. 2011). STAMBP-deficient mice, which are neonatal lethal by ~ day 19, develop post natal growth retardation associated with severe neuronal apoptosis in the hippocampus and cerebral cortex atrophy (Matsuoka et al. 2007; Ishii et al. 2001). These findings suggest that STAMBP-deficiency has a particular and profound impact upon normal cortical structure and function.

I examined a panel of LBLs derived from unrelated MIC-CAP patients for defects in STAMBP-associated cellular functions to try and identify novel cellular defects in this syndromal context that could help explain the clinical presentation of this disorder. In particular, loss of STAMBP in MIC-CAP resulted in accumulation of ubiquitin-conjugated protein aggregates and elevated apoptosis, which can be a mechanism influencing progressive neuronal loss, underlying microcephaly and severe neurodegeneration. Additionally, insensitive activation of the RAS-MAPK and RTK-PI3K-AKT-mTOR pathways in these cells can relate to the established association between these networks and capillary malformations, suggesting MIC-

CAP can be regarded as a *RASopathy*, although the consequences of STAMBP-deficiency are wider than the RAS-MAPK pathway alone.

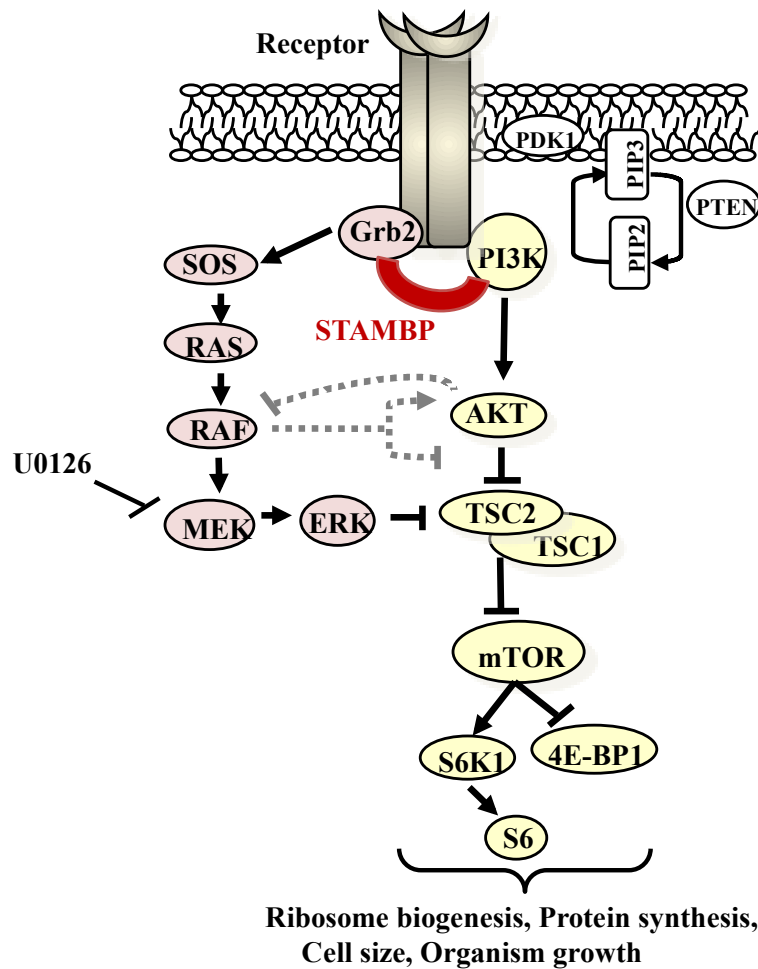


Figure 6.1.4. Schematic illustration of the RAS-MAPK and PI3K-AKT pathways.

As well as interacting with the ESCRT machinery and STAM, STAMBP has been shown to interact with other important components of several signal transduction pathways including the Grb2 adaptor and the class II PI3-kinase catalytic subunit.

6.2 Results

6.2.1 MIC-CAP patient cell lines exhibit low or absent STAMBP/AMSH protein expression

The relative levels of STAMBP protein expression in exponentially growing wildtype and MIC-CM patient cells were determined by western blotting, using anti-STAMBP (H-4) with an epitope directed to amino acids 131-270 (Santa Cruz Biotechnology).

STAMBP expression was not detected in MIC-CAP patients 7.1 and 1.2 (Figure 6.2.1a). In patient 7.1 only one mutation in *STAMBP* was identified, but analysis by western blot failed to detect STAMBP expression (Figure 6.2.1a) suggesting that P7.1 has MIC-CAP secondary to an undetected deletion or noncoding mutation. Patient 3.1 showed a reduced level of STAMBP expression compared to control cells, likely secondary to the Arg424* mutation being targeted for nonsense-mediated decay. (Figure 6.2.1b) No coding mutations were found in patient 5.1 using exome sequencing. The depth of coverage across the exons of *STAMBP* did not suggest a deletion and analysis of SNP (single nucleotide polymorphism) data was normal according to Laura McDonald (Ottawa, Canada). However, Western blotting revealed a severe reduction in STAMBP expression, (Figure 6.1.2c) suggesting that P5.1 has MIC-CAP secondary to noncoding (likely intronic) mutation in *STAMBP*.

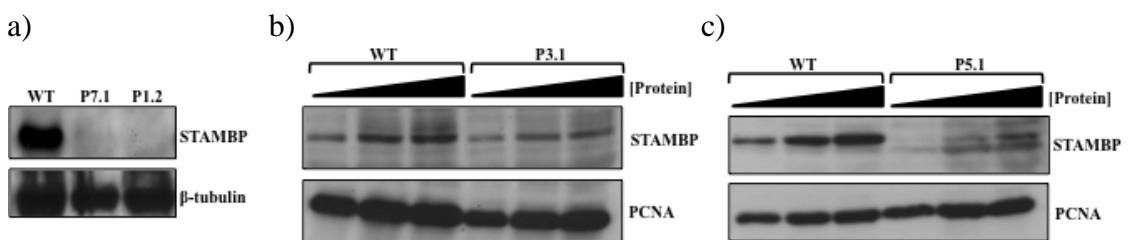


Figure 6.2.1. AMSH/STAMBP protein expression in MIC-CAP patient cell lines

Western blot analysis of whole cell extracts (WCE) from patient-derived LCLs P3.1, P5.1, P7.1 and P1.2, showing either **a)** equivocal (P3.1), **b)** reduced (P5.1) or **c)** absent STAMBP expression (P7.1, P1.2) when compared to WT cells. Increasing amounts of WCE (25μg, 50μg, 75μg) were blotted for WT versus P3.1 and WT versus P5.1, whilst 100μg of WCE were used to investigate STAMBP expression in P7.1 and P1.2. Expression of β-tubulin or PCNA was used as a control for protein loading.

Notably, patients 1.2 and 7.1, where no detectable STAMBP expression was found, seem to be associated with higher severity of some of the MIC-CAP phenotypical traits, namely lowest birth OFC, lowest later OFC and lowest birth length (Table 6.1.1) when compared to MIC-CAP patients with reduced STAMBP expression. This may be reflective of hypomorphic defects in this disorder.

6.2.2. STAMBP depletion results in accumulation of conjugated ubiquitin

Impaired ESCRT function is associated with the intracellular accumulation of ubiquitinated proteins (Peretz et al. 2001; Agromayor and Martin-Serrano 2006). Specifically, overexpression of catalytically inactive STAMBP has been shown to cause accumulation of ubiquitin at the endosomes (McCullough, 2004). Importantly, brain lesions containing ubiquitinated protein aggregates have been noted in *Stambp*^{-/-} mice, associated with severe atrophy of cerebral cortex and loss of hippocampal neurons (Huang-Doran et al. 2011; Suzuki et al. 2011; Ishii et al. 2001)

Consistent with this, I observed elevated levels of conjugated-ubiquitin aggregates following siRNA-mediated silencing of *STAMBP* in the human medullablastoma line T98G using indirect immunofluorescence (IF) with an antibody that specifically detects conjugated-ubiquitin (FK2) and not free ubiquitin (Figure 6.2.2).

Strikingly, elevated levels of conjugated-ubiquitin aggregates were also observed in several STAMBP patient LCLs, compared to wild-type (WT) controls following serum starvation (Figure 6.2.3).

Accumulation of ubiquitin aggregates as a result of loss of STAMBP function in neurons is likely a mechanism that could influence the development of microcephaly and its progression in MIC-CAP.

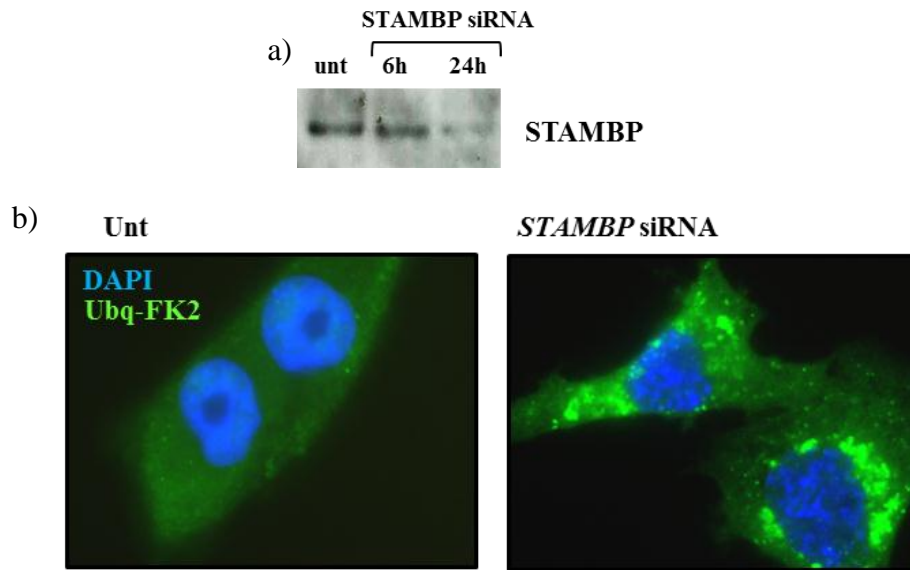


Figure 6.2.2. Elevated conjugated-ubiquitin protein aggregates were observed following siRNA-mediated silencing of STAMBP.

a) siRNA-mediated knockdown of *STAMBP* in T98G medulloblastoma cells was confirmed by western-blot (unt = untransfected). **b)** 24h post-transfection, the cells were immunostained with anti-FK2 antibody and a FITC conjugated secondary antibody (green) and ubiquitin aggregates visualized by indirect immunofluorescence (IF). DAPI counterstain (blue) provides nuclear staining. 100X magnification. *STAMBP* siRNA resulted in accumulation of ubiquitin aggregates in T98G cells.

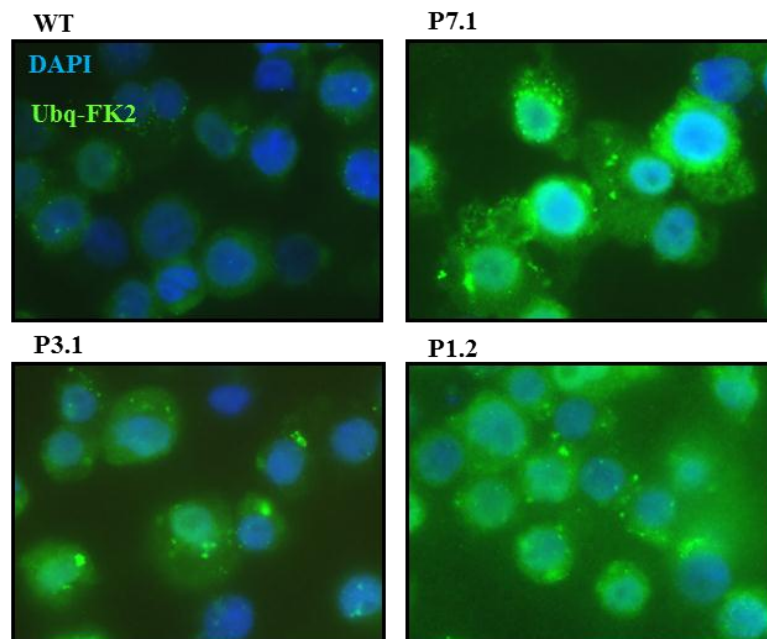


Figure 6.2.3. STAMBP-patient LCLs exhibit elevated levels of conjugated-ubiquitin protein aggregates.

WT and MIC-CAP patient derived LBLs were immunostained with anti-FK2 antibody and a FITC conjugated secondary antibody (green) and ubiquitin aggregates visualized by indirect immunofluorescence (IF). DAPI counterstain (blue) provides nuclear staining. 100X magnification. IF using anti-FK2 showed elevated levels of ubiquitinated protein aggregates in LCLs from P7.1, P3.1 and P1.1, compared to WT following 24hrs serum starvation.

6.2.3 MIC-CAP patient LBLs exhibit elevated autophagy flux

Endocytic DUBs including STAMBP are associated with the regulation of autophagy (Puc et al. 2005; Kirkin, McEwan, et al. 2009; Korolchuk, et al. 2009). Autophagosomes are formed as a catabolic response under conditions of nutrient limitation or for targeting expended/damaged cellular structures for lysosomal degradation.

Post-translational modifications of cell proteins (such as ubiquitination) provide a selective degradation signal for the autophagy pathway (N. Xu et al. 2010; Kirkin, et al. 2009; N. Xu et al. 2012). p62 and NBR1 (neighbor of BRCA1 gene 1) are autophagy receptors that bind both ubiquitin and autophagosome-specific modifiers LC3/GABARAP, and are thought to be involved in autophagy of ubiquitinated targets (Carter et al. 2011; Kirkin, et al. 2009; Isidor et al. 2011; Komatsu et al. 2007; Mirzaa et al. 2011).

During starvation-induced autophagy, the cytosolic form of microtubule-associated protein light chain 3 (LC3-I) is cleaved at its carboxy-terminal, and conjugated to phosphatidylethanolamine to form LC3-II. The lipid-conjugated form LC3II is associated to autophagosomal membranes (Kabeya et al. 2000). LC3 immunofluorescence did not reveal a clear difference in LC3 subcellular distribution/levels between MIC-CAP LBLs and the control (WT) (Figure 6.2.4a). However, studies have shown that the total autophagic flux is better assessed by immunoblot analysis of the ratio between the two forms of LC3 (LC3-II/LC3-I) (Mizushima and Yoshimori 2007) which correlates with the number of autophagosomes. Bafilomycin A1 inhibits fusion between autophagosomes and lysosomes. I examined processing of the autophagy marker LC3-I to LC3-II to evaluate autophagosome formation in STAMBP-deficient MIC-CAP cell lines. Treatment with the autophagy inhibitor Bafilomycin A1 helps monitor the autophagic flux, evidenced by an increase in LC3-II isoform expression in all LBL lines. Calculation of the LC3-II/LC3-I (cleaved/full length LC3A) ratio suggests an accumulation of autophagosomes in MIC-CAP LBLs, compared to WT levels, even in the untreated as well as after BafA1 treatment. This indicates upregulated autophagosome formation STAMBP-deficient MIC-CAP LBLs.

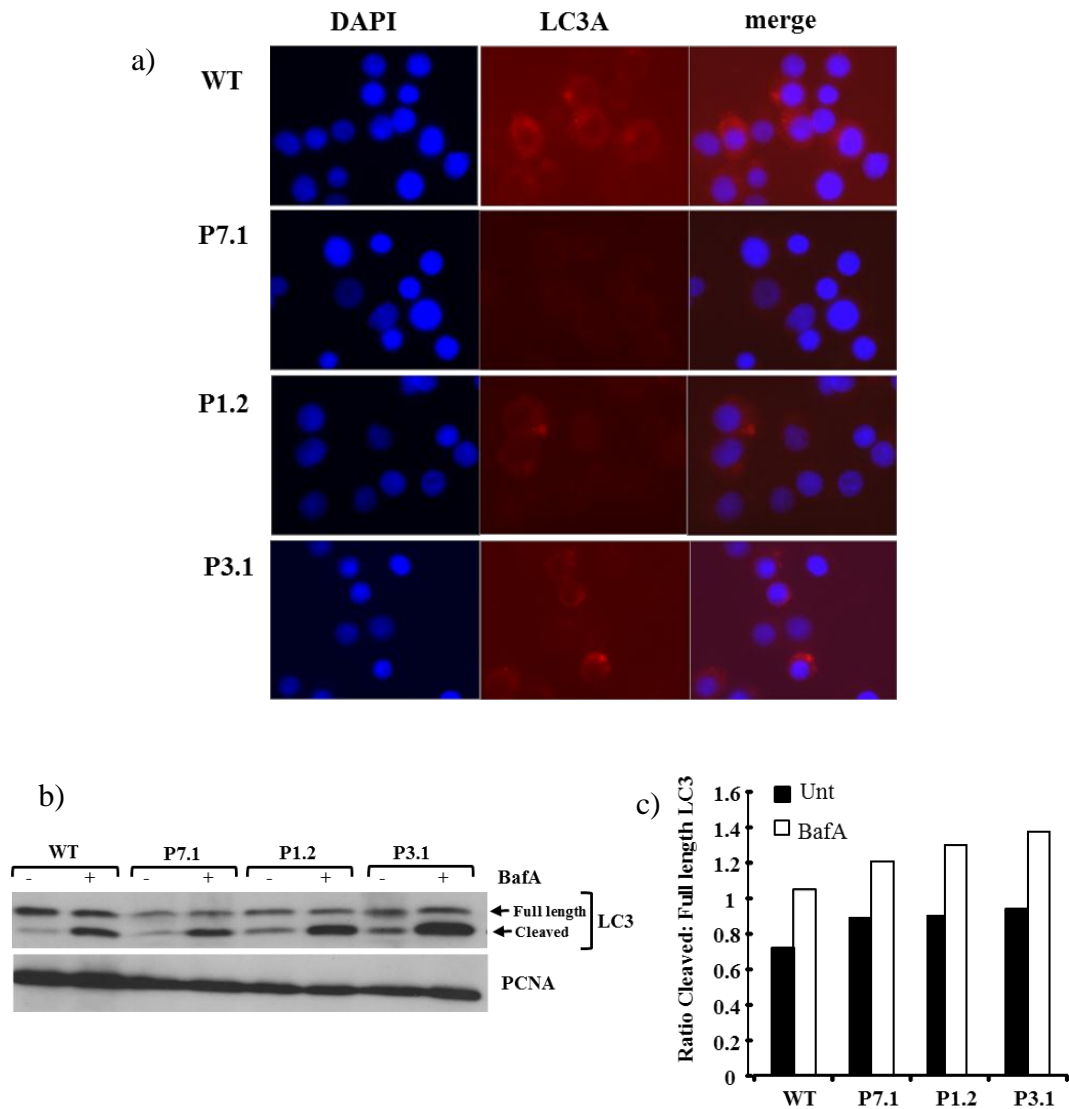


Figure 6.2.4. MIC-CAP patient LBLs show upregulated autophagosome formation.

The presence of LC3 in autophagosomes and the conversion of LC3 (full length) to the lower migrating form LC3-II (cleaved) were investigated to evaluate autophagy in MIC-CAP LBLs. **a)** WT and MIC-CAP patient derived LBLs were serum-starved for 24h to induce autophagy, and immunostained with autophagosome marker anti-LC3A antibody and a rhodamine-conjugated secondary antibody (red). DAPI counterstain (blue) provided nuclear staining. 100X magnification. Subcellular localization of LC3A by indirect immunofluorescence revealed a punctate cytoplasmic distribution, which did not visibly differ between WT and MIC-CAP cells. However, **b)** treatment with the autophagy inhibitor Bafilomycin A1 monitors autophagic flux, evidenced by an increase in LC3-II isoform expression in all LBL lines. WT and MIC-CAP LBLs were grown in the absence (-) or presence (+) of BafA1 for 2h, and cell lysates analyzed by western blot for the presence of LC3A. PCNA levels were used as a control for protein loading. **c)** calculation of the LC3-II/LC3-I (cleaved/full length LC3A) ratio reveals an accumulation of autophagosomes in MIC-CAP LBLs, compared to WT levels, even in the untreated as well as after BafA1 treatment.

6.2.4. MIC-CAP patient LBLs exhibit elevated apoptosis

Apoptosis has been shown to have an important role during normal mammalian brain development, namely involved in the regulation of forebrain size (Haydar et al. 1999). An increase in neuronal apoptosis has been linked to microcephaly in *Mos*^{+/-} mice (Silver et al. 2010; Schipani et al. 2001; Provot and Schipani 2007; Schipani 2006), whereas apoptosis-deficient *Caspase 9* knockout mice exhibit increased brain size (Schipani et al. 2001; Kuida et al. 1998), implying that a carefully regulated level of apoptosis is important for normal neural development. Importantly, increased apoptotic cell death in the cerebral cortex has been observed in a human disorder characterized by microcephaly, epilepsy and infantile diabetes (MEDS; OMIM 614231). MEDS results from a homozygous mutation in the gene *IER3IP1* (immediate early response 3 interacting protein 1), which codes for an endoplasmic reticulum stress response protein highly expressed in the fetal brain cortex (Poulton et al. 2011).

I assessed apoptosis in MIC-CAP patient LBLs, as a possible mechanism that could explain the severe microcephaly manifested in this disorder. Elevated levels of cleaved caspase-3, activated during the early stages of apoptosis, were observed specifically in the 3 MIC-CAP LBLs, by comparison to WT, upon 24h serum starvation-induced apoptosis (Figure 6.2.5a). Under the same conditions, a higher number of cells stained positively for annexin V in MIC-CAP patient LBLs, compared to WT (Figure 6.2.5c). These results indicate elevated apoptosis in MIC-CAP. Elevated ubiquitin-conjugated protein aggregation resulting from STAMBP deficiency could induce progressive apoptosis, and provide a potential underlying mechanism for microcephaly in this disorder. This would be consistent with brain imaging analysis of MIC-CAP (Carter et al. 2011), as well as *Stambp*^{-/-} mouse model, which showed that STAMBP is required for survival of hippocampal and cerebral cortex neurons (Lee et al. 2012; Ishii et al. 2001).

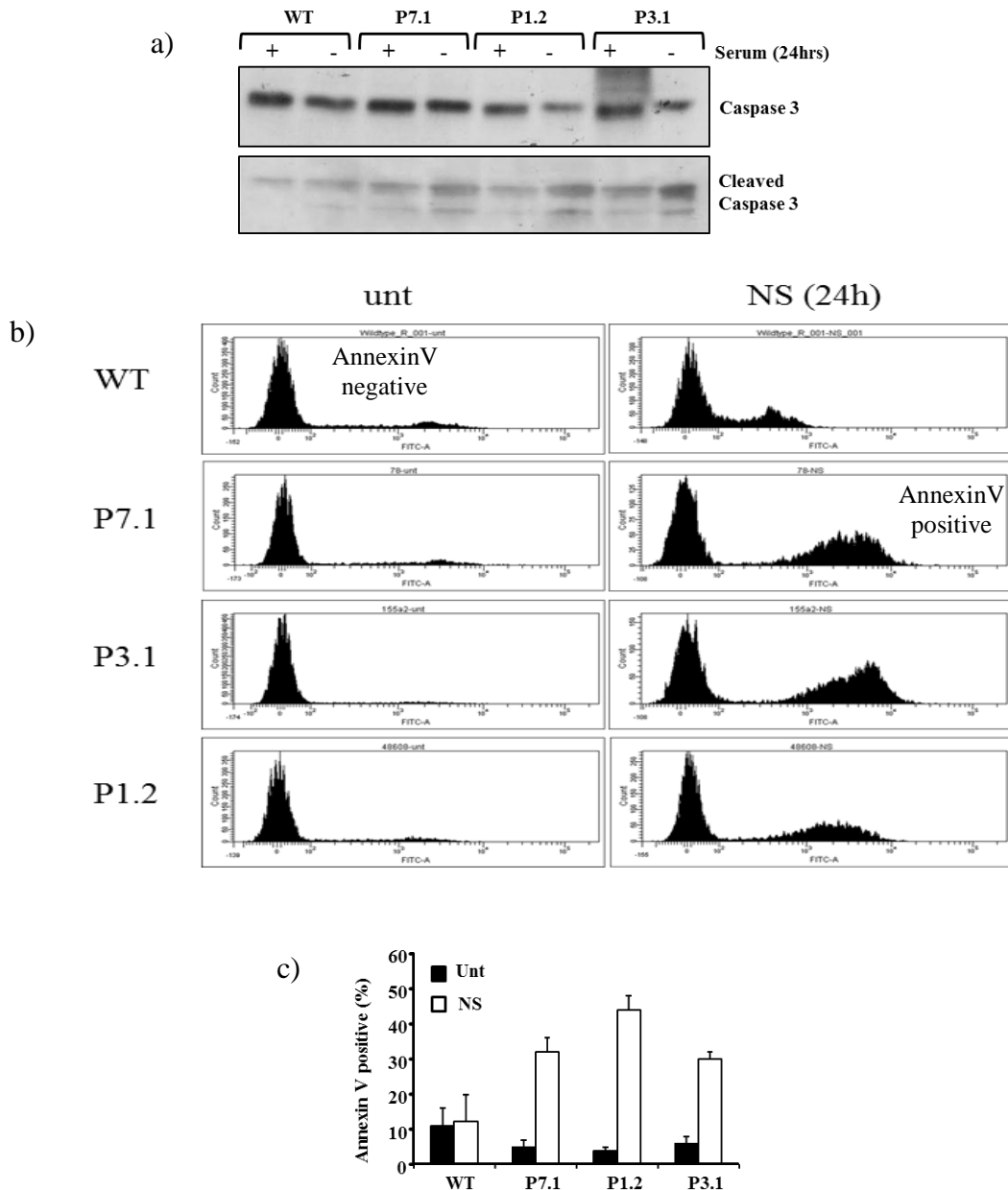


Figure 6.2.5. MIC-CAP patient LBLs exhibit elevated apoptosis.

WT and MIC-CAP LBLs were serum starved for 24h to induce apoptosis. **a)** western-blot analysis revealed an increased amount of (apoptotic marker) cleaved caspase 3 in LCLs from P7.1, P3.1 and P1.1, compared to WT, following 24hrs serum starvation, suggesting elevated apoptosis in MIC-CAP cells. Caspase 3 levels were used as loading control. Additionally, **b)** annexin V staining under the same conditions was also determined by flow cytometry (using the Single Channel Annexin V/Dead Cell Apoptosis Kit with AlexaFluor® 488 annexin V and SYTOX® Green for Flow Cytometry, by Invitrogen). Serum-starved cells showed a higher percentage of apoptotic cells than the basal level of apoptosis seen in the untreated. **c)** Moreover, MIC-CAP LBLs showed higher levels of annexin V staining, when compared to control (WT), in response to serum starvation. Unt; untreated, NS; no-serum 24h. Average of 3 separate determinations \pm SD. These results indicate that apoptosis is higher in STAMBP-defective MIC-CAP LBLs, when compared to WT levels.

6.2.5 Is MIC-CAP a RASopathy?

When the MIC-CAP syndrome was first described, the authors postulated that “the causative gene for this new syndrome may have a dual role in signaling pathways related to vasculogenesis and neuronal survival” (Carter et al. 2011).

Considering the established role of STAMBP in regulating receptor-mediated endocytosis, sorting and trafficking (Kyuuma et al. 2006; Clague et al. 2006) I investigated RAS-MAPK signal transduction in MIC-CAP LBLs, since mutations in components of this pathway are associated with congenital capillary malformation disorders (Chang et al. 2006; Zenker 2011; Tidyman et al. 2009). RAS-MAPK signal deregulation could help explain the capillary malformations observed in MIC-CAP patients.

RAS is a small GTP-binding protein (GTPase) that cycles between an inactive (GDP-bound) and an active (GTP-bound) form to regulate signal transduction pathways (Chang et al. 2006; Zenker 2011). GTP-bound RAS binds RAF-1, leading to activation of the MAPK pathway. Therefore, RAF-1 can be used as an affinity ligand to selectively precipitate GTP-RAS. To examine the direct effect of STAMBP-deficiency on RAS-MAPK pathway activation, I used a RAS pull-down assay to investigate the endogenous levels of active RAS in MIC-CAP LBLs relative to WT (Figure 6.2.6). Compared to WT levels, both P7.1 and P1.2 LBLs exhibited elevated levels of GTP-bound RAS. This suggests MIC-CAP syndrome is associated with hyperactive RAS.

Additionally, I investigated RAS-dependent signaling to the MAPK pathway, to determine if RAS over activation translates into perturbed regulation of this signaling cascade in MIC-CAP. Serum starvation induced a significant reduction in C-RAF phosphorylation on serine 338 in WT LBLs, consistent with inhibition of C-RAF activity under these conditions (Figure 6.2.7a). However, STAMBP-patient LBLs maintained pS338-C-RAF levels in the absence of serum indicating persistent activation and insensitivity of this pathway.

Further evidence suggesting insensitive signal transduction in this pathway in STAMBP-mutated LBLs is indicated by the relative insensitivity of these cells to the MEK1/2 inhibitor U0126. Active C-RAF phosphorylates and activates MEK1/2 kinase, which then phosphorylates and activates ERK1/2 (Figure 6.1.4). I repeatedly found elevated levels of phosphorylated ERK1/2 in exponentially growing

STAMBP-mutated LBLs compared to WT following a short treatment (1hr) with the MEK1/2 inhibitor U0126 (Figure 6.2.7b). Treatment with increasing doses of MEK1/2 inhibitor further confirmed this observation, revealing an increased residual level of phosphorylated ERK1/2 in MIC-CAP LBLs, when compared to control levels in the WT (Figure 6.2.7c). The excess of phosphorylated ERK1/2 in STAMBP-mutated LBLs under these robust inhibition conditions is consistent with hyperactive and insensitive RAS-MAPK pathway in these cells.

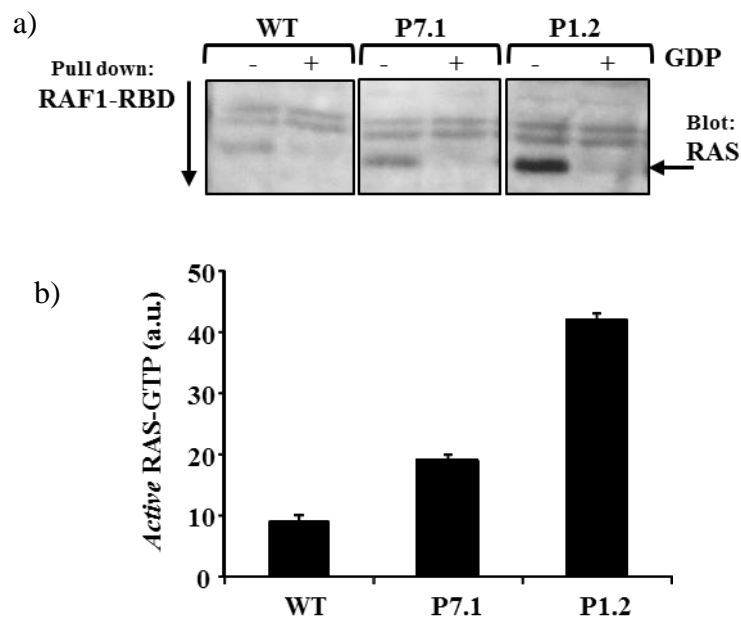


Figure 6.2.6 MIC-CAP LCLs exhibit hyperactive RAS

A RAS pull down assay was performed using the Ras Activation Assay Kit (Millipore). Briefly, WT and MIC-CAP LBLs lysates were incubated with RAF-1 RBD agarose to precipitate activated GTP-bound RAS. **a)** Western blot analysis was performed using anti-RAS antibody. GDP-loaded samples were used as a negative control. **b)** Measurement of the band intensities using Image J showed significantly elevated active RAS in MIC-CAP LBLs (P7.1, P1.2). (a.u., arbitrary units). This suggests MIC-CAP syndrome is associated with hyperactive RAS signaling.

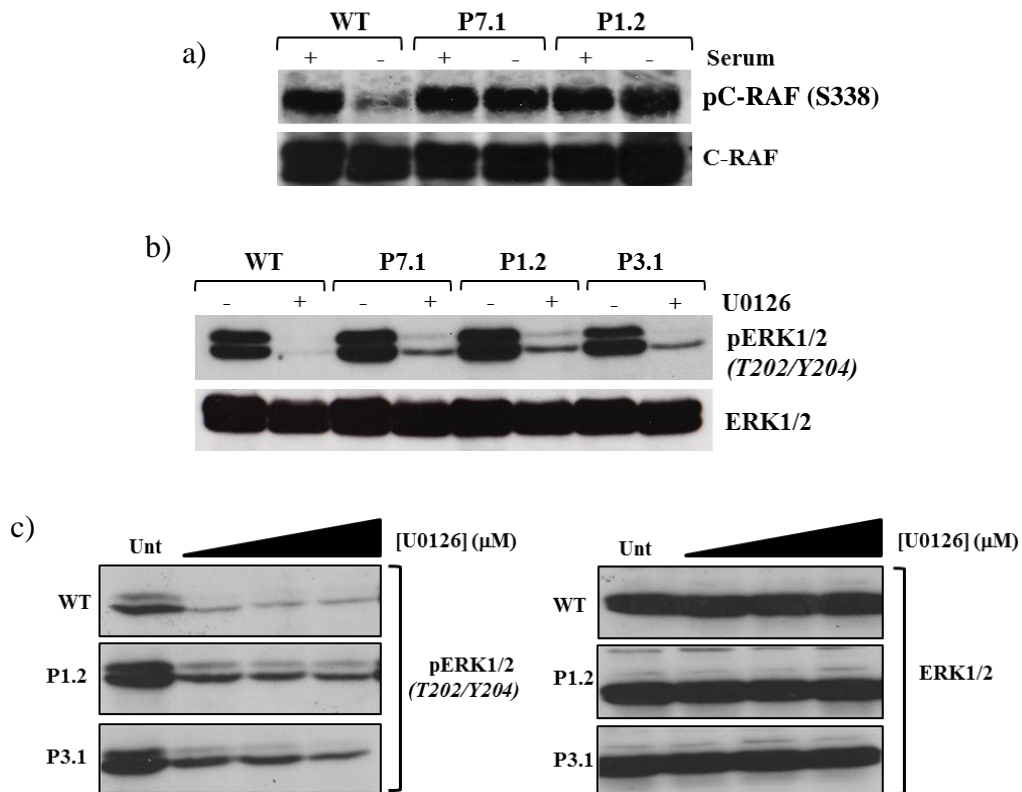


Figure 6.2.7 MIC-CAP LBLs exhibit active and insensitive RAS-MAPK signaling.

a) WT and MIC-CAP LBLs were grown in the presence (+) or absence (-) of serum for 24h. Serum starvation inhibits C-RAF activation (pS338 C-RAF) in wild type (WT) LBLs. In contrast MIC-CAP LBLs show inappropriately active C-RAF following serum starvation. **b)** LBLs were either treated (+) or untreated (-) with 10μM U0126, a potent and specific MEK1/2 inhibitor for 1hr. Cells were harvested and whole cell extracts were probed for phospho-ERK1/2 levels, which is mediated by MEK. MIC-CAP LBLs show residual pERK1/2 signal following MEK1/2 inhibition, reflecting an increased intensity of signal transduction from RAF to MEK to ERK. **c)** U0126 titration further confirms this phenotype, showing a residual pERK1/2 signal in MIC-CAP LBLs treated with increasing doses of inhibitor, when compared to WT levels. Collectively, these results indicate a greater strength RAS-MAPK signaling in MIC-CAP LBLs.

The RAS-MAPK pathway regulates crucial cellular processes including cell growth, cell cycle progression and differentiation. Disorders characterized by hyperactivity of RAS-MAPK network, such as Noonan and Costello syndromes, also exhibit growth delay, another prominent feature of MIC-CAP, alongside the microcephaly and capillary malformation (Cancer Genome Atlas Research Network 2008; Adams, et al. 2006; Tidyman et al. 2009). Considering the marked post-natal growth retardation and capillary abnormalities seen in MIC-CAP, hyperactive RAS-MAPK signaling may represent a significant biological consequence induced by impaired STAMBP function in humans and suggests that *STAMBP*-mutated MIC-CAP is a RASopathy.

6.2.6 MIC-CAP patient LBLs exhibit insensitive, elevated PI3K-AKT signaling

Chronically activated signaling via cell surface receptors (due to STAMP deficiency) would also be associated with chronically activated signaling through PI3K (phosphoinositol-3-kinase) and consequently through AKT. The PI3K-AKT pathway represents another crucial receptor-mediated signaling cascade controlling the cell's translational response to its environment (Lee et al. 2012; Gregory et al. 2004; Laplante and Sabatini 2012).

Here I showed direct evidence of spontaneously elevated PI3K activity in *STAMBP*-mutated LBLs that was not responsive to serum removal (NS) (Figure 6.2.8a). Phosphorylated PI3Kinase signal decreased in the WT following serum removal consistent with shutting off this pathway. However, both the p55 and the p85 isoform of PI3K were hyperphosphorylated specifically in MIC-CAP cells compared to WT, suggesting chronic activation of receptor-mediated signaling. Consistently, I saw elevated levels of activated, phosphorylated AKT in MIC-CAP patient cell lines, under the same conditions (Figure 6.2.8b).

Both the RAS-MAPK and PI3K-AKT pathways are interdependent (Lindhurst et al. 2012; Ruzankina et al. 2007; Castellano et al. 2010) and involve complex cross-talk between the networks at many levels, particularly at the RAF↔AKT interface (Figure 6.1.4.) ultimately converging on inhibition of the tuberous sclerosis complex (TSC1/2) via phosphorylation of tuberin (TSC2). The TSC1/2 complex is the principal negative regulator of mTORC1, which when active, enables ribosome biosynthesis and protein translation. I monitored the consequences of apparently increased signaling through the PI3K-AKT network by examining TSC2 phosphorylation on threonine-1462, which is an AKT-dependent *inhibitory* signal for the TSC1/2 complex. Under nutrient-limiting conditions, this site should be unphosphorylated, allowing active TSC1/2 to be able to inhibit mTOR function and thereby suppress protein translation. Consistent with this, pT1462-TSC2 levels are reduced in WT in no-serum conditions. In MIC-CAP cells, however, the TSC complex fails to be inactivated (Figure 6.2.8b). Maintenance of phosphorylated TSC2 under these conditions indicates inappropriate mTORC1 activation.

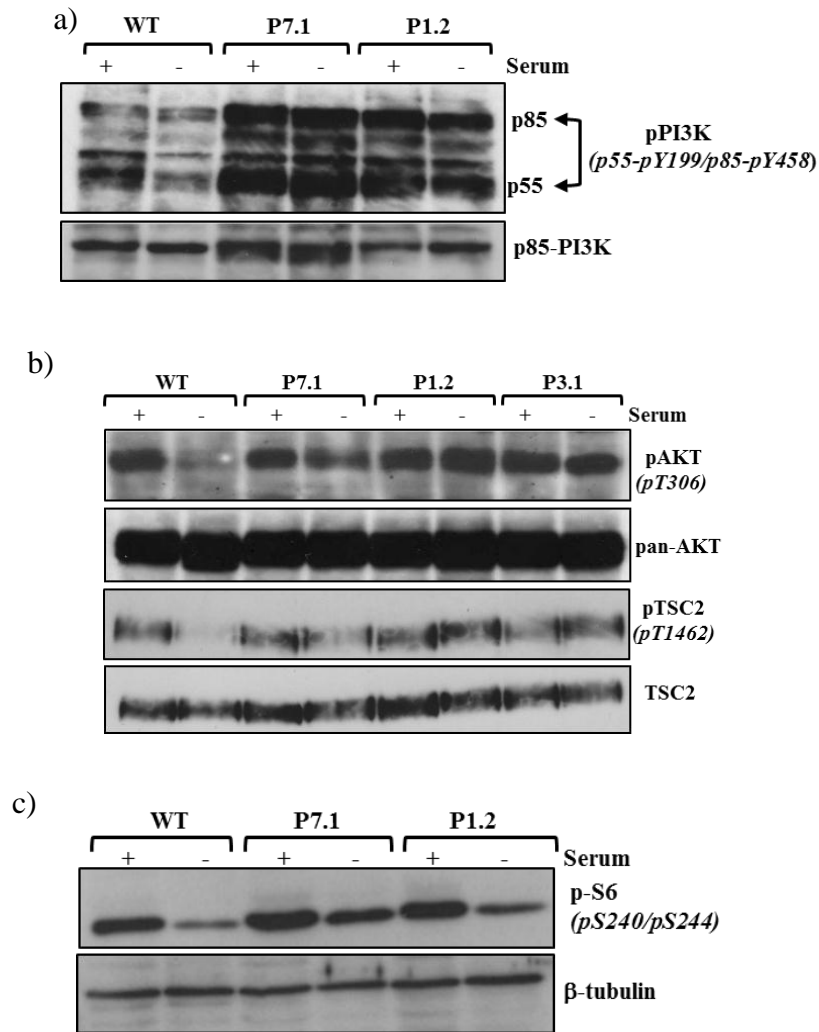


Figure 6.2.8. MIC-CAP LBLs exhibit insensitive, elevated PI3K-AKT signaling.

WT and MIC-CAP LBLs were grown in the presence (+) or absence (-) of serum for 24h. **a)** MIC-CAP LBLs from P7.1 and P1.2 exhibit elevated basal levels of *active* phosphorylated PI3K (p55-pY199/p85-pY458) when cultured in complete medium (15% FCS), compared to WT levels. Upon serum starvation (-) a modest reduction in p85 and p55 phosphorylation was seen in WT LBLs, consistent with attenuation of PI3K-mediated signaling, whereas both MIC-CAP LBLs consistently exhibited elevated levels of phosphorylation indicative of insensitive, hyperactive PI3K. The lower panel shows a re-probe of the above blot using antibodies against the native p85 PI3K regulatory subunit. **b)** MIC-CAP LBLs from P7.1, P1.1 and P3.1 exhibit elevated *active* phosphorylated-AKT (pT308) following serum starvation, compared to wild-type cells (WT). Similarly elevated levels of *inhibited*, AKT-dependent phosphorylated-TSC2 (pT1462) are observed in MIC-CAP LBLs following serum starvation, in contrast to WT cells. These results point to an increased activation of the PI3K/AKT/mTOR signalling in MIC-CAP cell lines, which is further supported by **c)** elevated mTOR-dependent phosphorylation of S6 (pS240/pS244) in STAMBP-deficient cells, compared to WT levels. pS6 is also less efficiently inhibited in serum-deprived cultures, compared to WT. β-tubulin levels were here used as a control for protein loading.

Moreover, in MIC-CAP LBLs, mTORC1-dependent phosphorylation of S6 (S240/S244) is elevated when compared to WT (Figure 6.2.8c), and in response to serum-starvation STAMBP-deficient LBLs fail to efficiently inhibit S6 activation. Activated mTOR pathway is likely to have causal impacts on clinical features such as growth retardation, manifested in MIC-CAP syndrome.

Collectively, these results indicate that MIC-CAP causative mutations in *STAMBP* are associated with insensitive, elevated signal transduction in both the RAS-MAPK and PI3K-AKT pathways in patient derived cells.

6.2.7. *STAMBP* reintroduction rescues MIC-CAP cellular phenotypes.

To demonstrate that the novel cellular phenotypes described here in MIC-CAP patient LBLs are the direct consequence of *STAMBP* deficiency, I reintroduced *STAMBP* in MIC-CAP cells using a lentiviral vector-mediated approach (LentiORF, Thermo Scientific Open Biosystems). Transduced P1.2 LBLs, where previously no *STAMBP* protein was detected, successfully re-expressed *STAMBP* to levels comparable to WT LBLs (Figure 6.2.9a). This resulted in correction of several cellular MIC-CAP phenotypes. A reduction of ubiquitin conjugated protein aggregates was now observed in *STAMBP*-complemented P1.2, as visualized by FK2 immunofluorescence (Figure 6.2.9b). *STAMBP* complementation also rescued signaling defects observed in MIC-CAP. Namely, under nutrient limiting conditions, *STAMBP*-expressing P1.2 LBLs efficiently downregulate signaling through the RAS/MAPK - as evidenced by pS338-C-RAF reduction- as well as through the AKT/mTOR pathway, suggested by dephosphorylation of pT1462 TSC2 as well as p240/244 S6, thus behaving like the control (WT) cells. This is in stark contrast to MIC-CAP response, which exhibits elevated, insensitive signaling via these pathways. *STAMBP* re-introduction also restored autophagosome levels in a MIC-CAP LBL, indicated by the reduction in LC3II/LC3I ratio compared to WT (Figure 6.2.9d).

Expression of *STAMBP* in a MIC-CAP patient-derived LBL lead to correction of multiple cellular MIC-CAP phenotypes, indicating that these defects resulted from the absence of *STAMBP* in these cells

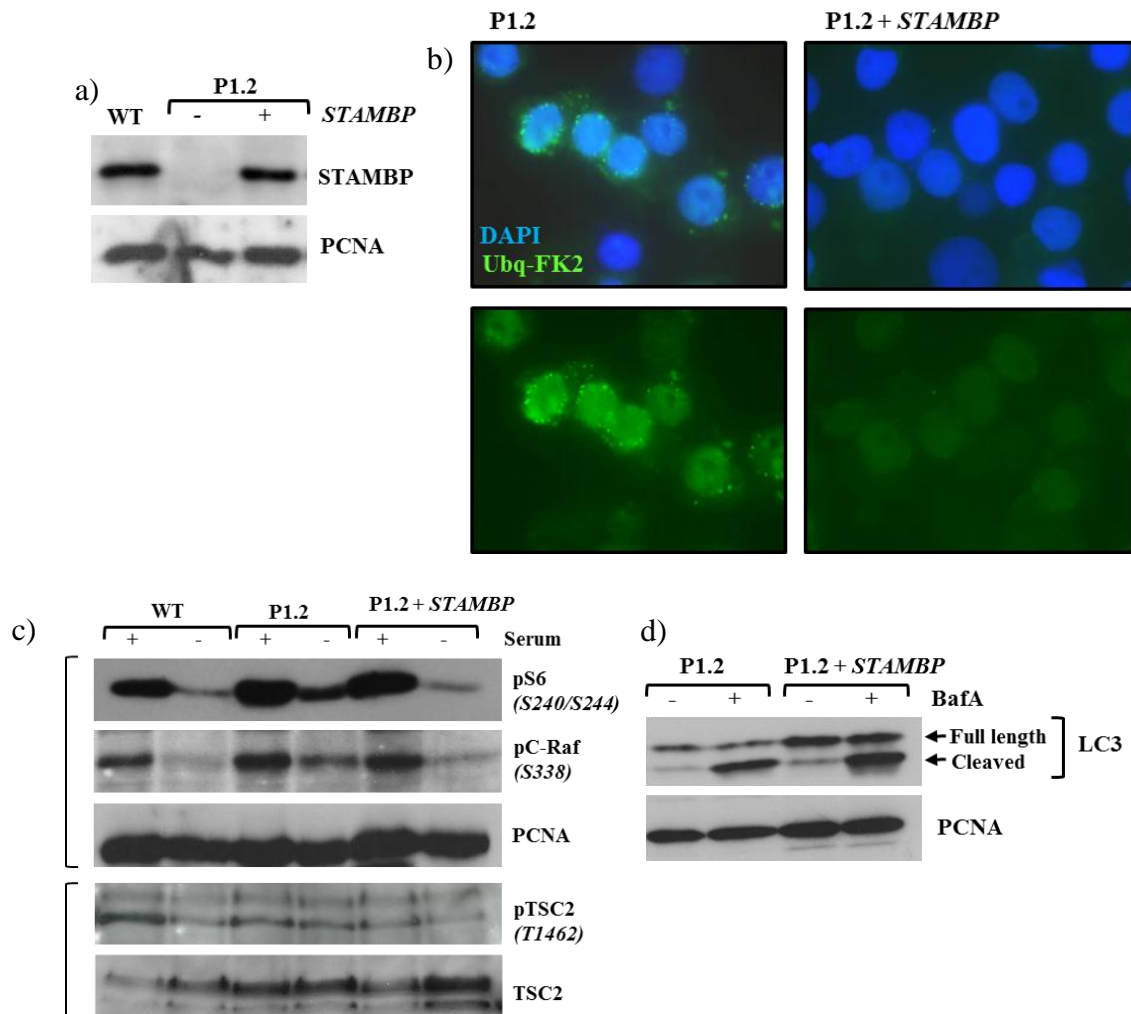


Figure 6.2.9. STAMBP-complementation rescues specific cellular MIC-CAP phenotype.

a) STAMBP-complementation of a MIC-CAP LBL line (P1.2), where no STAMBP expression was originally detected, was confirmed by western blotting. Complemented LBLs express STAMBP to levels comparable to WT. **b)** STAMBP complementation rescued the accumulation of ubiquitin aggregates observed in MIC-CAP cells. Following 24hrs of serum starvation, P1.2 and P1.2+STAMBP LBLs were immunostained with anti-FK2 antibody and a FITC conjugated secondary antibody (green) and ubiquitin aggregates visualized by indirect immunofluorescence (IF). DAPI counterstain (blue) provided nuclear staining. 400X magnification. **c)** STAMBP complementation in P1.2 also rescued cell signaling defects observed in MIC-CAP cells. Intrinsically active and insensitive signaling via the PI3K-AKT and RAS-MAPK pathway pathways observed in MIC-CAP LBLs was rescued in P1.2 by complementation with STAMBP. Western blot analysis showed reduced phosphorylation of S6 (pS240/pS244), C-RAF (pS338) and TSC2 (pT1462) after 24h serum starvation in P1.2-STAMBP complemented cells, by contrast to parental P1.2, and similar to WT LBLs. **d)** Additionally, analysis of LC3A-II/LC3A-I ratio after Bafilomycin A treatment revealed also a reduction in autophagy flux after STAMBP complementation in P1.2 MIC-CAP LBLs. PCNA levels were used as control for protein loading. Collectively, this data indicates that the cellular defects observed in MIC-CAP patient derived LBLs are the result of STAMBP deficiency in these cells.

6.2.8 MIC-CAP patient derived LBLs show inefficient ATR-dependent G2/M checkpoint.

Microcephaly (and growth retardation) is a clinical feature often observed in human disorders that manifest with DNA damage response defects (Kurek et al. 2012; French et al. 2004; O'Driscoll et al. 2008). Examples include Seckel Syndrome (OMIM 210600), Microcephalic Osteodysplastic Primordial Dwarfism type II (OMIM 210720), Primary Microcephaly and Meier-Gorlin Syndrome (OMIM 224690). This suggests that efficient DNA damage response is important during normal brain development and can be an underlying aetiology of microcephaly. Additionally, increased AKT-dependent signaling (such as seen in MIC-CAP) has been shown to overcome cell cycle checkpoints and induce mitosis in the presence of DNA damage (Rios et al. 2012; French et al. 2004; Kandel et al. 2002); and in Chapter 5 I presented evidence of a microcephalic disorder, Donohue syndrome, associated with increased PI3K-AKT signaling which manifests with an ATR-dependent cell cycle checkpoint defect. For these reasons, I investigated some basic aspects of the DNA damage response in MIC-CAP patient derived LBLs to determine whether there was any crosstalk between a defective endocytic DUB and a functional cell cycle checkpoint.

Fascinatingly, I found that MIC-CAP LBLs fail to activate the ATR-dependent G2/M cell cycle checkpoint in response to UV-induced damage (Figure 6.2.10a), compared to WT. *STAMBP*-mutated LBLs efficiently induced the IR-induced checkpoint arrest. This suggests impairment specifically of the ATR-related (but not ATM) G2/M checkpoint in MIC-CAP. However, and unlike in Donohue syndrome, treatment with different doses of a specific AKT inhibitor did not rescue the checkpoint defect in MIC-CAP LBLs (Figure 6.2.10b). This suggests that the DDR defect in *STAMBP*-mutated cells does not derive from increased AKT activation in these cells. Nonetheless, the observation of a defective ATR-dependent G2/M checkpoint arrest in cells deficient in an endosome-associated DUB represents a novel functional association that warrants deeper investigation.

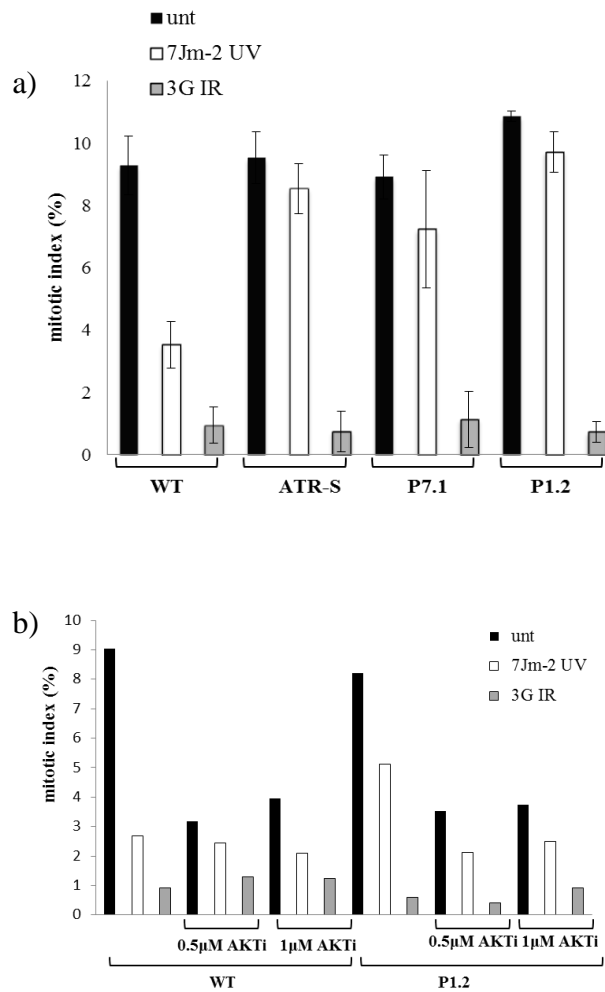


Figure 6.2.10. MIC-CAP patient LBLs present defective ATR-dependent G2/M checkpoint activation.

a) Control (WT), ATR-defective (ATR-S) and MIC-CAP (P7.1, P1.2) LBLs were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and the number of mitotic cells assessed 4hrs later. ATR-S, and both MIC-CAP patient derived cells failed to efficiently activate the ATR-dependent G2/M checkpoint arrest, compared to control cells. By contrast, the IR-induced (ATM dependent) checkpoint was efficiently activated under these conditions. Mean ± SD of 3 experiments.

b) AKT inhibition does not rescue the ATR-dependent G2/M DNA damage checkpoint defect in MIC-CAP. Treatment with different doses of AKT inhibitor (InSolution™ AKT Inhibitor IV, cat.124015, Calbiochem) resulted in decreased cell proliferation in both WT, as well as MIC-CAP (P1.2), even in un-irradiated cells. This suggests that the checkpoint defect observed in these patient cells is not due to the observed elevated levels of active AKT in MIC-CAP. Data represents a single determination.

6.3 Summary

In summary, *STAMBP* mutations were identified in MIC-CAP syndrome, a recently described severe developmental disorder characterized by microcephaly, growth retardation and capillary malformations. Analysis of MIC-CAP patient LBLs demonstrated elevated ubiquitin-conjugated protein aggregation and apoptosis activation. This data is consistent with elevated ubiquitin-conjugated protein aggregate induced progressive apoptosis as a potential underlying mechanism for microcephaly in this disorder. Furthermore, I observed active and insensitive RAS-MAPK pathway and PI3K-AKT pathway signaling as a novel consequence of defective *STAMBP*, which could potentially contribute to the vasculature defects and growth characteristics of MIC-CAP syndrome. Finally, MIC-CAP patient derived LBLs exhibit defective ATR-dependent DDR, a common feature of microcephalic disorders, which could be an additional important contributor to the microcephaly in this syndrome, although the molecular basis of this defect is currently completely unclear. This work presents an example of a human disorder caused by a congenitally defective endosome-associated DUB, providing significant new insights into the pathophysiology of human microcephaly and capillary malformation.

6.4 Discussion

In this chapter I described the identification and functional characterization of novel *STAMBP* mutations in a cohort of children with a severe congenital developmental and neurodegenerative disorder: microcephaly-capillary malformation syndrome (MIC-CAP). Using Western blotting analysis I observed absent or severely reduced *STAMBP* expression in MIC-CAP patient derived LBLs.

STAMBP-mutated MIC-CAP syndrome is associated with severe microcephaly, intractable seizures, post-natal growth retardation and capillary malformations (Kandel et al. 2002; Matsubara et al. 1992; Mirzaa et al. 2011; Carter et al. 2011; Isidor et al. 2011). Interestingly, *Stambp*^{-/-} mice are viable and exhibit post-natal growth retardation with severe neurological impairment and death between post-natal days 19-23 (O'Driscoll et al. 2007; Araldi and Schipani 2010; Suzuki et al. 2011). The brain abnormalities in these mice are region specific, typified by cerebral neuron loss and degradation of the CA1 hippocampal layer with relative sparing of the cerebellar cortex and olfactory bulbs (Stankiewicz et al. 2010; Suzuki et al. 2011; Colnaghi et al. 2011; Ishii et al. 2001). This is in keeping with the diffuse cortical and hippocampal atrophy with cerebral sparing described in MIC-CAP patients (P1.2, P3.1 and P7.1 in Table 6.1.1; Figure 6.1.1a,c).

STAMBP encodes a deubiquitinating enzyme (DUB) that plays a central role in controlling the sorting and trafficking of ubiquitinated endocytosed protein cargo (Colnaghi et al. 2011; McCullough et al. 2006; McCullough, et al. 2004; Tanaka et al. 1999; Suzuki et al. 2011). Impaired endosomal sorting complex required for transport (ESCRT) function is associated with the intracellular accumulation of ubiquitinated proteins. Specifically, overexpression of catalytically inactive *STAMBP* has been shown to cause accumulation of ubiquitin at the endosomes (McCullough, et al. 2004) and brain lesions containing protein aggregates have been noted in *STAMBP*^{-/-} mice (Suzuki et al. 2011).

Here, I showed that siRNA-mediated knockdown of *STAMBP* resulted in accumulation of ubiquitin conjugated protein aggregates in T98G human medullablastoma cells. Strikingly, examination of MIC-CAP patient derived LBLs revealed elevated levels of ubiquitin-conjugated protein aggregates in these cells when compared to WT levels, following nutrient deprivation.

Autophagy is a key mechanism required for the degradation of expended and/or damaged proteins/macromolecules, and is up-regulated to provide nutrients during starvation (Mizushima et al. 2007). Much of the endosomal machinery is also associated with autophagy and indeed autophagosomes can fuse with multivesicular bodies (MVBs) and are regulated by ESCRT components. Impaired autophagy has been associated with the intracellular accumulation of ubiquitinated proteins. Deficient autophagic degradation of protein aggregates has been also associated with neuropathologies such as Alzheimer's, Parkinson's and prion disease (Ventruti and Cuervo 2007). Autophagic clearance of accumulated protein aggregates is therefore an important mechanism in preventing disruption of neural function and neurodegeneration.

I found STAMBP deficiency caused increased accumulation of autophagosomes in MIC-CAP LBLs, when compared to WT levels. This is consistent with the observation that depletion of certain ESCRT components does not prevent autophagosome formation, but instead inhibits the autophagic flux towards the lysosome (Lee et al. 2007; Rusten et al. 2008; Wright, et al. 2011). Interestingly, STAMBP interacts with a number of ESCRT-III components at late endosomal sub-compartments (Agromayor and Martin-Serrano 2006). Mutations in the ESCRT-III subunit *CHMP2B* (charged multivesicular body protein 2B, also known as chromatin-modifying protein 2B)/Vps2B), were found in patients exhibiting frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS). *CHMP2B*-mutations inhibited autophagic degradation and accumulation of autophagosomes in mammalian cells, also associated with increased levels of ubiquitin-positive protein aggregates (Stiehl et al. 2002; Rusten et al. 2008). The accumulation of ubiquitinated protein aggregates in MIC-CAP could be associated with impaired ESCRT-III function due to lack of STAMBP at the late endosomal pathway, which would also compromise efficient autophagy.

Careful regulation of apoptosis is crucial during brain development (Haydar et al. 1999), and its deregulation has been associated specifically with altered forebrain size. I observed elevated apoptosis in MIC-CAP LBLs under the same nutrient limiting conditions, when compared to control (WT) levels. Elevated apoptosis in neurons has been linked to microcephaly (Silver et al. 2010). Conversely, mice deficient in caspase 9 exhibit increased brain size (Kuida et al. 1998). Increased

apoptotic cell death in the cerebral cortex has also been observed in a distinct human disorder associated with microcephaly, epilepsy and infantile diabetes (Poulton et al. 2011). Importantly, STAMBP has been shown to be required for survival of hippocampal and cerebral cortex neurons (Ishii et al. 2001). Elevated ubiquitinated protein aggregation resulting from STAMBP deficiency could induce progressive apoptosis in neuronal cells, and provide a potential underlying mechanism for the development of microcephaly and its progression in MIC-CAP.

Endocytosis of activated cell surface receptors is a fundamental process controlling protein homeostasis and regulation of signal transduction duration and amplitude/magnitude (Raiborg et al. 2009). Considering the overt capillary defect seen in MIC-CAP patients, and the association between activating mutations in components of the RAS-MAPK pathway seen in related capillary disorders, I investigated RAS-MAPK pathway function in MIC-CAP. STAMBP-patient LBLs maintained pS338-C-RAF levels after serum starvation, indicating persistent activation and insensitivity of this pathway. Further evidence came from the relative insensitivity of MIC-CAP LBLs to the MEK1/2 inhibitor, U0126, which resulted in elevated levels of phosphorylated ERK1/2 in these cells, when compared to WT levels. *STAMBP*-mutated LBLs exhibit hyperactive RAS function, associated with active and insensitive RAS-MAPK signaling. The data presented here suggests that MIC-CAP is a novel *RASopathy* associated with progressive and pronounced neurodegeneration.

Activating *RAS* mutations have been well documented to drive oncogenic transformation and are frequently found in human tumours, associated with hyperactivation of the RAS-MAPK pathway, an important regulator of cell proliferation (Zenker et al. 2011). Indeed oncogenic *K-RAS* mutations are found in about 30% of all tumours, most commonly present in pancreatic ductal adenocarcinoma and significantly high percentages of lung and colon tumors. Mutations in *N-RAS* are prevalent in leukemias, thyroid carcinomas, or malignant melanoma (Fernández-Medarde et al. 2011). The stable but discrete increase in RAS-MAPK signaling observed in *RASopathies* (Tidyman et al. 2009) (Aoki et al. 2008) contrasts with the more aggressive, stronger and constitutively active signal produced by oncogenic RAS mutations. Still, *RASopathies* mediated by mutations in

components of the RAS-MAPK pathway are prone to developing tumors. For example, Noonan syndrome has been associated with K-RAS and N-RAS mutations (Schubbert et al. 2006; Cirstea et al. 2010). Activation of RAS-MAPK signal transduction resulting from these mutations is increased when compared to WT RAS activation, but to a lesser degree than observed in most cancers.

It is possible that the *STAMBP* mutations found in MIC-CAP, resulting in hyperactivation of the RAS-MAPK pathway, could increase the susceptibility of MIC-CAP patients to developing tumors, but none have yet been identified, although the molecularly defined cohort is still simply too small to enable a definite conclusion to be drawn.

Chronically activated signaling via cell surface receptors (due to STAMP deficiency) should also be associated with chronically activated signaling through PI3K (phosphoinositol-3-kinase) and consequently through AKT. I found evidence of hyperactive signaling in the PI3K-AKT pathway in MIC-CAP patient derived cells. The functional interplay between the RAS-MAPK and the PI3K-AKT pathways has been well established. Additionally, STAMBP interactants include key components of receptor signaling pathways including GRB2 and the Class II PI3-kinase catalytic subunit (Arts et al. 2011; Sowa et al. 2009; Tsang et al. 2006). Yet how this contributes to MIC-CAP patient phenotypes is unclear. Interestingly, intractable epilepsy, a specific feature found in all MIC-CAP patients, has been associated with enhanced mTOR signaling (Farquharson et al. 2000; Crino 2009). K63-linked polyubiquitination of the AKT kinase has been shown to promote localization of AKT to the cell membrane, and subsequently activation of AKT (O'Driscoll et al. 2003; G. Wang et al. 2012). These non-proteolytic functions of ubiquitination, including kinase activation, are also likely to be affected by loss of STAMBP DUB function, which normally functions by removing specifically K63-linked polyubiquitin chains.

Syndromes with dramatic PI3K-AKT pathway hyperactivity such as Cowden's disease, tuberous sclerosis and Peutz-Jehgers syndromes, are often associated with cell or organism overgrowth. This is in contrast to disorders characterized by pure hyperactivity of the RAS-MAPK network, such as Noonan and Costello syndromes (Tidyman et al. 2008). Considering the marked post-natal

growth and capillary abnormalities seen in MIC-CAP, hyperactive RAS-MAPK signaling may represent the predominant biological consequence induced by STAMBP deficiency in humans. Nevertheless, elevated PI3K-AKT-mTOR has also been implicated in vascular abnormalities, so a contribution of this pathway cannot be formally excluded, or indeed must be included (Griffith et al. 2008; Castellano et al. 2011; Karar et al. 2011).

The precise mechanism by which loss of STAMBP DUB function can result in increased receptor mediated signaling is yet to be elucidated. Indeed contradictory studies can be found in the literature. McCullough et al observed an increased rate of EGFR degradation in *STAMBP* knockdown cells ((McCullough et al. 2004) On the other hand, catalytically inactive STAMBP has been shown to inhibit degradation of endosomal cargo (Agromayor et al. 2006).

Interestingly, inhibition of STAMBP targeting to endosomes has been shown to prevent degradation, but not internalization, of EGFR (Ma et al. 2007). It is increasingly clear that endocytosis and signal transduction cannot be viewed as separate processes but instead exhibit a great level of cross talk. The established view is that endocytosis terminates receptor mediated signaling by removing active receptors from the cell membrane. Internalized receptors and their factor are targeted to the MVBs and the lysosomal pathway for degradation (Williams et al. 2007); alternatively, endocytosed ubiquitinated receptors can be recycled back to the cell surface.

Recently, endosomes have also been viewed as potential signaling platforms, maintaining or even promoting specific signals (Dobrowolski et al. 2012; Sorkin et al. 2009). Internalized receptors can remain active on the surface of early endosomes (signaling endosomes), potentially increasing the duration and intensity of receptor dependent signaling. This has been proposed for MAPK-mediated signaling since all components of this signaling cascade have been found in the endosome (Alderton et al. 2004; Sorkin et al. 2009). The net effect of endosomal signaling would be dependent of how rapidly or efficiently the activated receptors are sorted to the degradative compartments. In this model, slow or impaired receptor sorting at the endosome due to loss of STAMBP function could result in sustained or increased receptor-mediated signal transduction from the signaling endosome, aggregation of ubiquitinated cargo and subsequent apoptotic cell death.

Finally, *STAMBP*-mutated MIC-CAP is also associated with defective ATR-dependent G2/M checkpoint activation. Specifically, MIC-CAP patient derived LBLs show impaired ATR-dependent (but not ATM dependent) activation of the G2-M cell cycle checkpoint. Microcephaly is a feature commonly observed in human disorders associated with defective DNA damage response (Mochida et al. 2001; O'Driscoll et al. 2008), suggesting that the regulation of DNA damage response is essential for normal brain development, and therefore may play a role in the development of microcephaly also in MIC-CAP syndrome.

Moreover, increased AKT activation has also been reported to surpass cell cycle checkpoints and promote entering into mitosis following DNA-causing damage (Murga et al. 2009; Kandel et al. 2002). However, the mechanism underlying the checkpoint defect in MIC-CAP is not associated with hyperactive AKT observed in these cells, as treatment with a specific and potent AKT inhibitor failed to rescue the checkpoint defect. Similarly, RAS signalling has been implicated in the DNA damage response (Ruzankina et al. 2007; Gladfelter 2010). But preliminary data using MEK inhibitor (not shown) doesn't seem to point to a hyperactive RAS-MAPK mediated effect as the causative agent behind the defective checkpoint activation in MIC-CAP LBLs. Therefore, the mechanism of *STAMBP*-mediated checkpoint defect remains elusive but clearly suggests a previously unknown route to cell cycle dysfunction.

The role of *STAMBP* in endocytosis has been primarily researched however, *STAMBP* contains a functional NLS and a substantial *STAMBP* pool has been shown to localize to the nucleus. This suggests additional, yet unclear *STAMBP* functions in the nucleus, which can potentially be relevant for DNA repair pathways. Nevertheless, MIC-CAP syndrome potentially highlights an important functional interplay between RTK-mediated signal transduction-endocytosis and the ATR-dependent DNA damage response.

In conclusion, the work described in this chapter is the first example of a human disorder caused by a congenitally defective DUB isopeptidase functioning in the endocytic pathway, providing significant new insights into the pathophysiology of human microcephaly and capillary malformation.

Chapter Seven

Results V: Mutations in the PI3K/AKT pathway genes AKT3, PIK3R2 and PIK3CA cause a spectrum of related megalencephaly syndromes

7.1 Introduction

To further investigate the role of PI3K-AKT signaling in brain, limb and vascular development, in this chapter I will characterize aspects of the PI3K-AKT-mTOR function in two megalencephaly disorders harboring novel mutations in core components of this pathway.

Megalencephaly-capillary malformation (MCAP; OMIM 602501) and megalencephaly-polymicrogiria-polydactyly-hydrocephalus (MPPH; OMIM 603387) syndromes are two recently described developmental disorders characterized by marked brain overgrowth. The anomalies observed in MCAP patients include primary megalencephaly - defined as more than 2 standard deviations above the mean for the patients age and gender (Matsuoka et al. 2007; DeMyer 1986), prenatal overgrowth, brain and body asymmetry, cutaneous vascular malformations, digital anomalies (syndactyly with or without postaxial polydactyly) and connective tissue dysplasia (Ruzankina et al. 2007; Mirzaa et al. 2012; Mirzaa et al. 2004). MPPH is distinguished from MCAP in that it lacks the vascular malformations, as well as syndactyly (Figure 7.1.1, Table 7.1.1). Common complications of both syndromes include hydrocephalus, seizures, and cerebellar tonsillar ectopia that may lead to Chiari malformation (abnormal lowering of cerebellar tonsils below the foramen magnum) with or without syringomyelia (development of a cyst in the spinal cord) (Karsenty 2008; Mirzaa et al. 2012). Additionally, the literature suggests a moderately higher incidence of cancer in MCAP and MPPH patients, as well as occurrence of benign tumors (Table 7.1.2).

Detailed clinical analysis of 42 children with MCAP or MPPH revealed significant phenotypic overlap with Noonan, cardiofaciocutaneous and Costello syndromes (i.e. high incidence of relative macrocephaly, vascular malformations, connective tissue dysplasia), which are caused by dominant mutations in *RAS* pathway genes (Araldi and Schipani 2010; Mirzaa et al. 2012). This suggested that MCAP and MPPH may be associated with altered *RAS* pathway function.

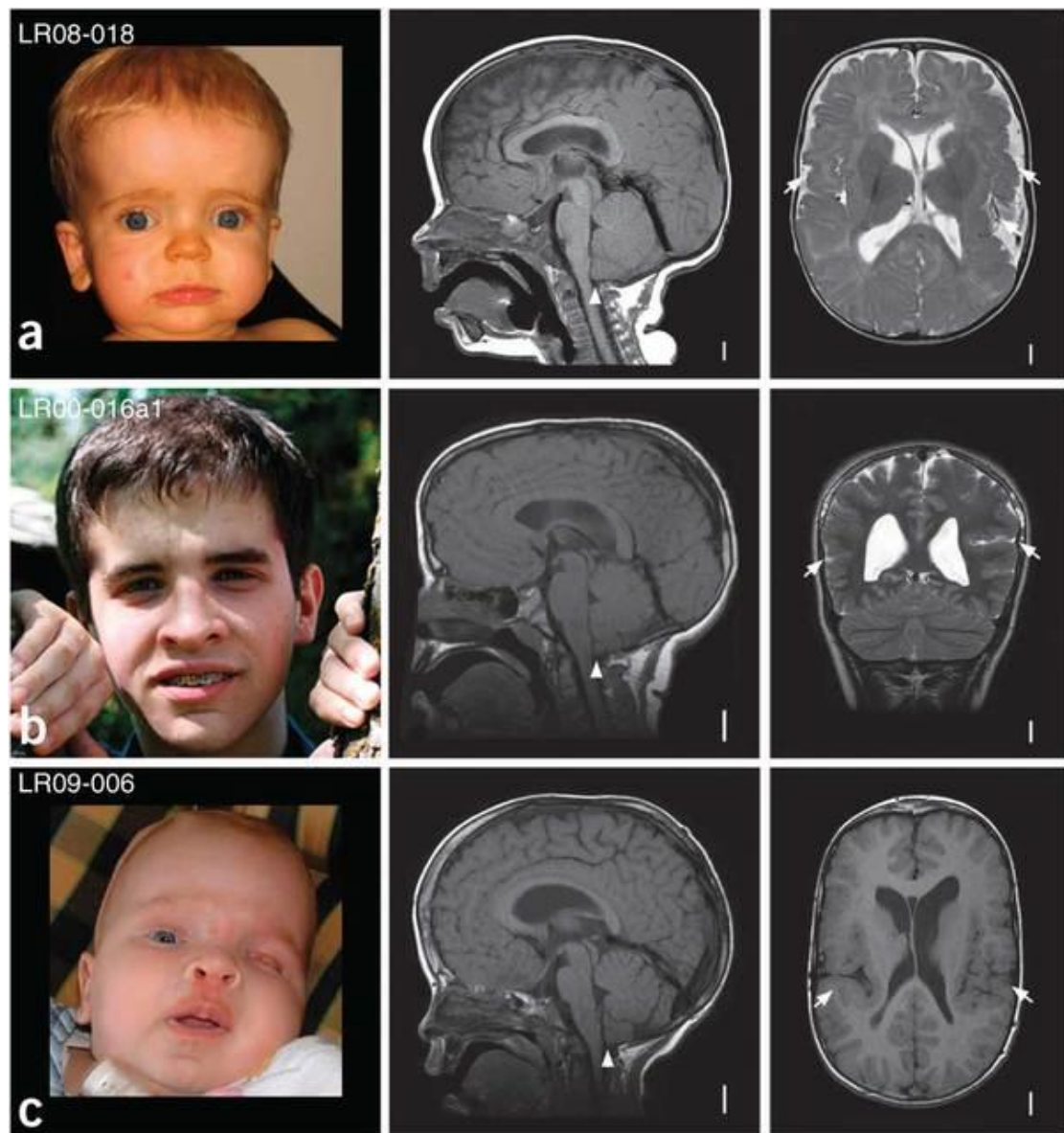


Figure 7.1.1. Craniofacial appearance and magnetic resonance imaging (MRI) of 3 megalencephaly subjects featured in this study.

Photos of patients LR08-018 (a), LR00-016a1 (b) and LR09-006 (c) were taken at 11 months, 15 years, and 5 months, respectively. Note the prominent forehead and apparent macrocephaly in all three patients, and a midline facial capillary malformation (or nevus flammeus) in LR09-006. On brain imaging, note a prominent forehead, increased cranium-to-face ratio, cerebellar tonsillar ectopia (arrow heads) and bilateral perisylvian polymicrogyria (arrows). Adapted from (Stiehl et al. 2002; Rivière et al. 2012)

Table 7.1.1. AKT3, PIK3R2 and PIK3CA mutations and clinical features of the megalencephaly patients featured in this study.

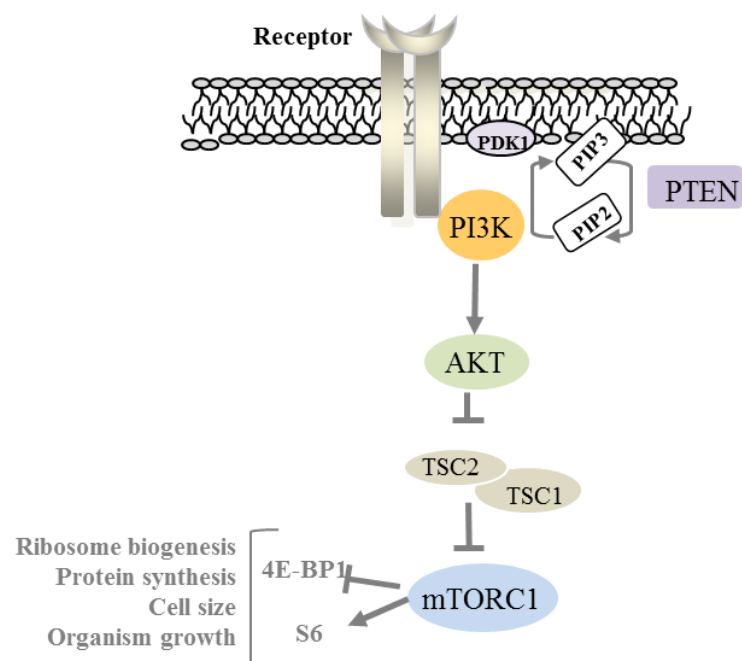
Patient ID	LR08-018	LR00-016a1	LR05-204	LR09-006
Group	Overlap	MPPH	MPPH	MCAP
Gene	<i>AKT3</i>	<i>PIK3R2</i>	<i>PIK3CA</i>	<i>PIK3CA</i>
cDNA change	c.1393C>T	c.1117G>A	c.1359_1361del	c.2740G>A
Amino acid change	p.Arg465Trp	p.Gly373Arg	p.Glu453del	p.Gly914Arg
sex	M	M	M	M
Age last assessed	11m	16y	4y	5y6m
Last OFC-SD (age)	+5.5 (7.5m)	+5 (13y)	+9-10 (4y)	+9-10 (5y)
OG/ASYM	nd/-	-	+/-	+/+
VM	+	-	-	+
SYN	-	-	-	+
POLY (hands/feet)	-	-	-	+
CONN	+	-	+	+
HYD/VMEG	+	+	+	+
CBTE	+	+	+	+
PMG	+	+	+	+

Abbreviations: ASYM, asymmetry; CBTE, cerebellar tonsillar ectopia; CONN, connective tissue dysplasia (including skin laxity, joint hypermobility, thick subcutaneous tissue); HYD, hydrocephalus; nd, no data; OFC, occipito-frontal circumference; OG, overgrowth; PMG, polymicrogyria; POLY, polydactyly; SD, standard-deviations; SYN, syndactyly; VM, vascular malformations; VMEG, ventriculomegaly. (adapted from (Phornphutkul, Wu, and Gruppuso 2006; Rivière et al. 2012))

Table 7.1.2 Reports of cancer in MCAP and MPPH

Subject	Syndrome	sex	age	Cancer type	Reference
<i>Malignant tumors</i>					
Patient 2	MCAP	F	10m	Wilms tumor	<i>Lapunzina et al</i>
Patient 3	MCAP	M	4y	Wilms tumor	<i>Wright et al.</i>
Not stated	MCAP	U	18y	Leukemia	<i>Moore et al</i>
Patient 2	MPPH	M	2y	Medulloblastoma	<i>Osterling et al.</i>
<i>Notable benign tumors</i>					
Not stated	MCAP	U	21m	Meningioma	<i>Moore et al.</i>
Patient 14	MCAP	M	5y	Meningioma	<i>Conway et al.</i>

Abbreviations: F, female; M, male; mo, months; U, unknown sex; y, years.

**Figure 7.1.2. Schematic illustration of the PI3K-AKT pathway.**

Several components of the PI3K/AKT signal transduction pathway (Figure 7.1.2) have been previously implicated in human overgrowth syndromes. MPPH and MCAP share clinical features with Proteus syndrome, recently found to be caused by an activating *AKT1* mosaic mutation (Marsh, et al. 2011; Lindhurst et al. 2011). Conversely, in mice, *Akt1* loss is associated with growth retardation and increased apoptosis (Cho et al. 2001). Also, a spectrum of related overgrowth disorders resulting from germline loss-of-function mutations in *PTEN*, a negative regulator of PI3K signaling, share several clinical features with MCAP and MPPH. These include Bannayan–Riley–Ruvalcaba syndrome, Cowden disease and autism with severe megalencephaly (Butler et al. 2005; Liaw et al. 1997; Marsh et al. 1998; Orrico et al. 2009).

In view of the phenotypic similarity between MCAP and MPPH, it was likely that the two disorders could be caused by mutations affecting the same pathway. Our collaborators, Prof. W. B. Dobyns (Seattle, USA) and Dr. Kim Boycott (Ottawa, Canada) used a combination of exome sequencing, Sanger sequencing, restriction fragment length polymorphism, and targeted ultra-deep sequencing in 50 families with MCAP or MPPH to identify the causative genetic defect. *De novo* germline or postzygotic mutations were identified in core components of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway (Rivière et al. 2012) (Figure 7.1.3).

A *de novo* mutation in *AKT3* was identified in a patient with clinical features overlapping MCAP and MPPH (subject LR08-018). Interestingly, *AKT3* encodes a supposedly brain-predominant form of the AKT serine/threonine kinase, a major downstream mediator of PI3K signaling. Sanger sequencing revealed a second *de novo* mutation in *AKT3* in a MPPH patient, suggesting that mutations in *AKT3* are a rare cause of megalencephaly. Significantly, an activating mutation in the kinase domain of *Akt3* (p.Asp219Val) has been associated with brain overgrowth and seizures in mice (Tokuda et al. 2011).

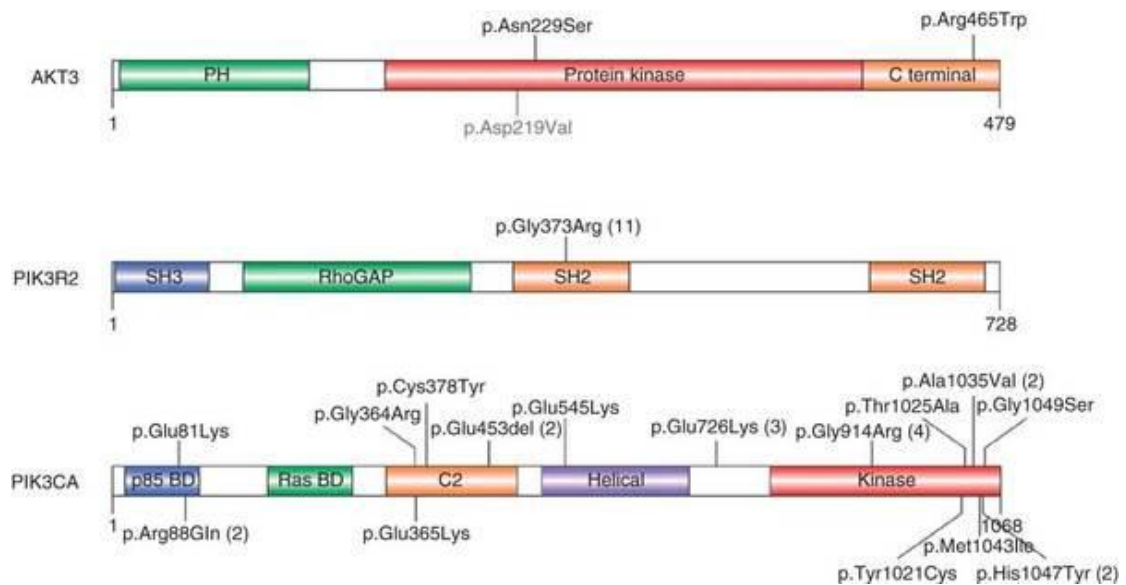


Figure 7.1.3. Distribution of AKT3, PIK3R2 and PIK3CA mutations found in MCAP and MPPH patients.

The activating *Akt3* mutation in mouse is indicated in grey (p.Asp219Val). For recurrent mutations, the number of occurrences in distinct patients is indicated in parentheses. PH: pleckstrin homology domain; C-terminal: carboxyl-terminal domain; SH2 and SH3: Src-homology-2 and -3 domains; RhoGAP: Rho GTPase-activating protein domain; p85-BD and RAS-BD: p85- and RAS-binding domains; C2: protein-kinase-C-homology-2 domain. The PIK3CA mutations affect a total of 15 residues, mainly localized in the p85-binding, C2, and catalytic lipid kinase domains. Adapted from (Rivière et al. 2012).

Furthermore, a recurrent mutation in *PIK3R2*, and multiple mostly postzygotic mutations in *PIK3CA* were further identified in MCAP and MPPH patients, which encode the p85 β regulatory subunit and the p110 α catalytic subunit of class IA PI3K, respectively. Mutations of all 3 genes led to pre and post-natal overgrowth of brain and variably other tissues (Rivière et al. 2012).

I examined a panel of MCAP and MPPH cell lines (Table 7.1.1) harboring mutations in *AKT3*, *PIK3CA* and *PIK3R2* for alterations in PI3K-AKT signaling that could help explain the clinical presentation of this disorder, and further our knowledge of the role of this signaling cascade in brain, limb and vascular development. Our working hypothesis based on the association of PIK3CA mutations in cancer was that all megalencephaly-causing mutations would result in *increased* activation of the PI3K-AKT-mTOR signal transduction pathway. To this end I designed functional assays to interrogate this possibility.

7.2 Results

7.2.1. Impact of *AKT3*, *PIK3R2* and *PIK3CA* mutations on PI3K activity in MCAP and MPPH patient LBLs.

Class IA PI3K dimers are composed of a p110 catalytic subunit and a p85 regulatory subunit, each of which has three isoforms encoded by three genes (p110 isoforms: *PIK3CA*, *PIK3CB* and *PIK3CD*; p85 isoforms: *PIK3R1*, *PIK3R2* and *PIK3R3*) (Engelman, Luo, and Cantley 2006). Interestingly, activating mutations in five of these six genes, particularly *PIK3CA* and *PIK3R1*, have been observed in many human cancers (Cancer Genome Atlas Research Network 2008). The sequencing data for MCAP and MPPH patients show that mutations in the PI3K p85 β (*PIK3R2*) regulatory and p110 α (*PIK3CA*) catalytic subunits are a common cause of megalencephaly syndromes, albeit with a clear genotype-phenotype correlation with *PIK3R2* and *PIK3CA* mutations being associated with MPPH and MCAP, respectively. The regulatory p85 subunits are known to stabilize the catalytic subunits, influence their localization and binding partners, and maintain them in a low activity state (Huang et al. 2007; Miled et al. 2007).

I found the *PIK3CA*, *PIK3R2* mutations in MCAP and MPPH patients do not result in significantly altered levels of protein expression for the different PI3K subunits (Figure 7.2.1.a,b) when compared to expression levels in the control (WT) LBL.

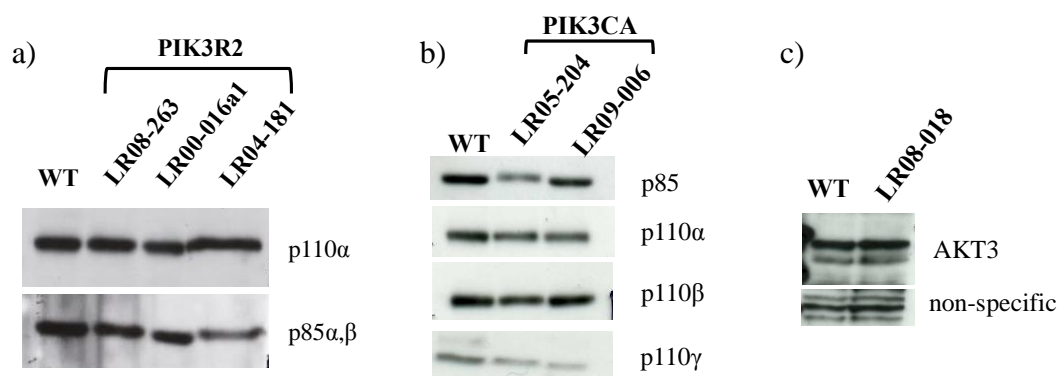


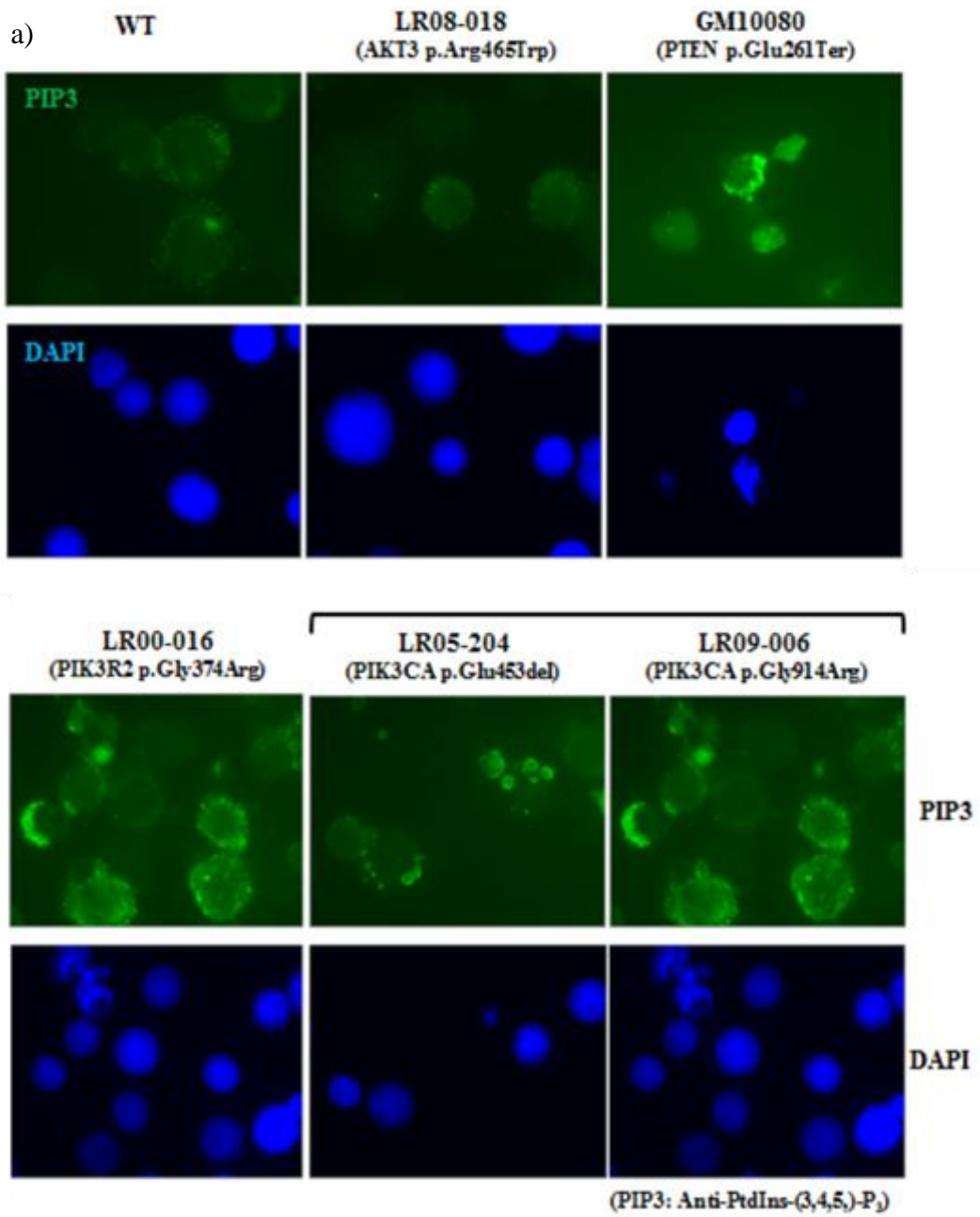
Figure 7.2.1. PI3K and AKT3 protein expression in different megalencephaly patient cell lines.

Western blot analysis of whole cell extracts (WCE) from **a)** *PIK3R2*-mutated; **b)** *PIK3CA*-mutated and **c)** *AKT3*-mutated patient-derived LBLs. The mutations found in these megalencephaly patients do not result in changed levels of protein expression for the various PI3K subunits analyzed **a)** and **b)**, as well as AKT3 isoform **c)**, when compared to control (WT) levels.

Similarly, *AKT3*-mutated megalencephaly patient LBLs express *AKT3* to levels comparable to those seen in wild type (WT) cells (Figure 7.2.1.c).

Both *PIK3R1* and *PIK3R2* have oncogenic potential, and mutations including the glycine-to-arginine substitution of *PIK3R2* found in MPPH (p.Gly373Arg) have been found in cancer (Cheung et al. 2011). Several of these mutations have been proposed to disrupt the inactive conformation of the PI3K dimer and maintain the catalytic subunit in a *high activity state* (Miled et al. 2007; Huang et al. 2007).

Class I PI3Ks convert phosphatidylinositol-4,5-bisphosphate (PIP₂) to phosphatidylinositol-4,5-trisphosphate (PIP₃), resulting in activation of downstream signaling components, notably AKT (Vanhaesebroeck et al. 2001) (Figure 7.1.3). In order to assess the impact of *AKT3*, *PIK3R2* and *PIK3CA* mutations on PI3K activity, I performed PIP₃ immunostaining using a monoclonal antibody to phosphatidylinositol 3,4,5-trisphosphate (PIP₃) developed by Caltag-MedSystems in lymphoblastoid cell lines derived from four megalencephaly mutation carriers (*AKT3* p.Arg465Trp, *PIK3R2* p.Gly373Arg, and *PIK3CA* p.Glu453del and p.Gly914Arg), and compared PIP₃ levels to those in control (WT) cells and a Cowden's disease line mutant in *PTEN* (Figure 7.2.2.a). *PTEN* is a phosphatase that specifically catalyzes the de-phosphorylation of PIP₃, counteracting the activity of PI3K, and therefore cells that are *PTEN*-deficient should have elevated levels of PIP₃. Similar to *PTEN* loss and consistent with elevated PI3K activity, all three *PIK3R2* or *PIK3CA* mutant lines showed significantly increased PIP₃ levels compared to unaffected control cells (Figure 7.2.2.b). I found no evidence for increased PI3K lipid kinase activity in the *AKT3* mutant line, which is consistent with a mutation affecting a downstream effector of PI3K. Decreased PIP₃ levels in the *PIK3CA* p.Glu453del mutant line upon treatment with a PI3K specific inhibitor (PI-103) confirmed that these observations are PI3K-dependent (Figure 7.2.3.). This data strongly supports and demonstrates for the first time increased activation of PI3K activity in *PIK3R2* and *PIK3CA* mutated megalencephaly patients.



b)

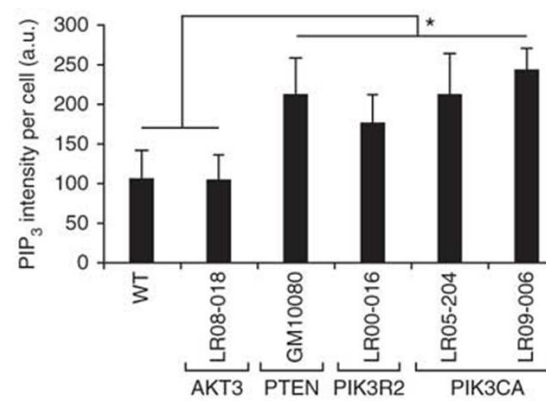


Figure 7.2.2. PIP3 levels are elevated in PI3K-mutated LBLs derived from megalencephaly patients.

Lymphoblastoid cell lines derived from an unaffected control (WT), a patient with Cowden disease (GM10080), and four megalencephaly patients. **(a)** Indirect immunofluorescence staining of PIP3 in exponentially growing lymphoblastoid cell lines using a mouse monoclonal anti-PIP3 antibody. **(b)** Per-cell quantification of PIP3 levels based on anti-PIP3 signal intensity (a.u., arbitrary units) using ImageJ. Levels of PIP3 signal in control cells (WT) are comparable to those of LR08-018 (*AKT3* p.Arg465Trp). All other mutant cell lines show increased PIP3 signal compared to control cells. Elevated PIP3 signal is also evident in cells derived from the patient with Cowden disease (*PTEN* p.Glu261Ter), which served as positive control. DAPI counterstain (blue) provides nuclear staining. 100X magnification. * Statistically significant difference compared to control cells ($p < 0.05$ two-tailed t-test assuming unequal variance, $n = 30$ to 50 cells per cell line). Error bars indicate standard deviation.

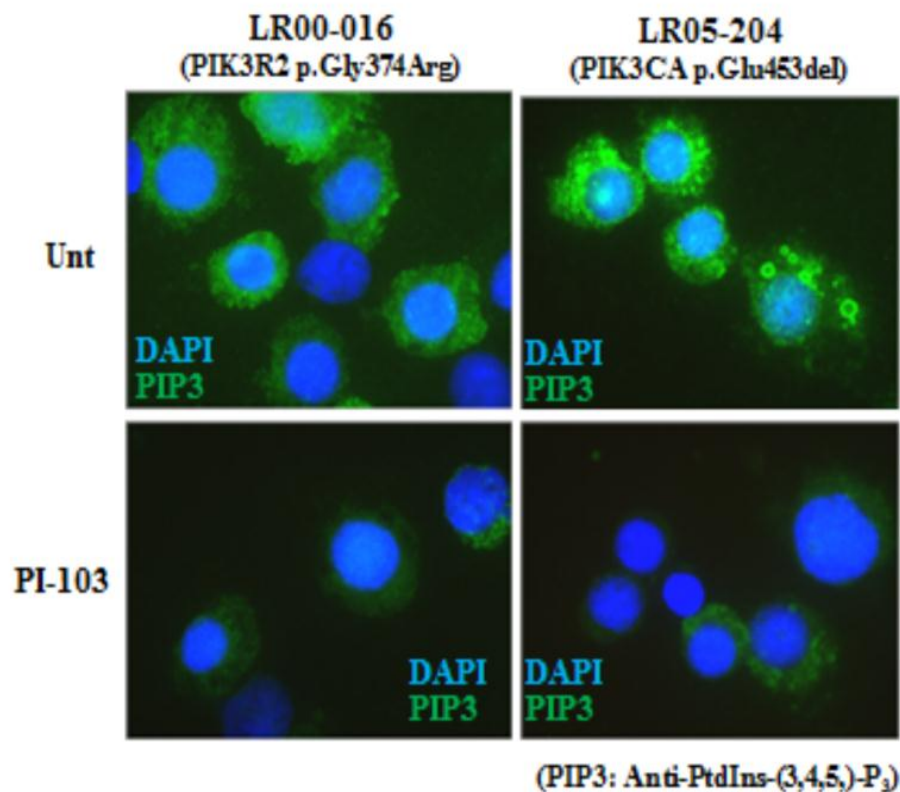


Figure 7.2.3. Levels of PIP3 in PI3K-mutated LBLs can be reduced by treatment with the PI3K inhibitor, PI-103.

Indirect immunofluorescence staining of PIP3 in exponentially growing lymphoblastoid cell lines using a mouse monoclonal anti-PIP3 antibody (green). Megalencephaly patient derived cells LR00-016a1 (*PIK3R2* p.Gly373Arg) and LR05-204 (*PIK3CA* p.Glu453del) were treated with the PI3K-inhibitor, PI-103 (5 μ M for 16 hours). DAPI counterstain (blue) provided nuclear staining. 100X magnification. PIP3 levels were efficiently reduced after treatment with the PI3K inhibitor, indicating that the PIP3 phenotype observed in these patient cells is PI3K-dependent.

7.2.2. *PIK3R2* and *PIK3CA*-mutated LBLs exhibit increased membrane-localized activated PDK1.

To further determine whether the *PIK3R2* and *PIK3CA* mutations found in MCAP and MPPH patients result in increased PI3K signaling, I investigated the localization of active phosphoinositide-dependent kinase 1 (PDK1) in patient-derived LBLs.

PDK1 interacts with membrane phospholipids such as PIP₃, generated by PI3-kinases, which promote PDK1 recruitment to the cell membrane. Membrane localization of PDK1 is required for activation of AKT on Thr308, a key step in the PI3K downstream signal transduction (K. E. Anderson et al. 1998). In un-stimulated cells, active, phosphorylated PDK1 is mainly found in the cytosol (Bayascas 2010; Bayascas 2008).

Indirect immunofluorescence staining in MCAP and MPPH patient derived lymphoblastoid cells shows increased localization of active p-PDK1 Ser241 at the cell membrane, a PI3K-dependent event (Figure 7.2.4) when compared to control (WT) cells. This was observed in both *PIK3CA* and *PIK3R3* mutated LBLs (LR00-016a1 (*PIK3R2* p.Gly373Arg), LR05-204 (*PIK3CA* p.Glu453del) and LR09-006 (*PIK3CA* p.Gly914Arg)), but not in the *AKT3*-mutated megalencephaly cell line ((LR08-018 p.Arg465Trp), which is consistent with a mutation occurring downstream of PI3K activation in *AKT3*-mutated cells. Increased membrane localized endogenous p-Ser241 in PI3K-mutated individuals further supports hyperactive PI3K activity in the PI3K-mutated MCAP and MPPH patient cell lines.

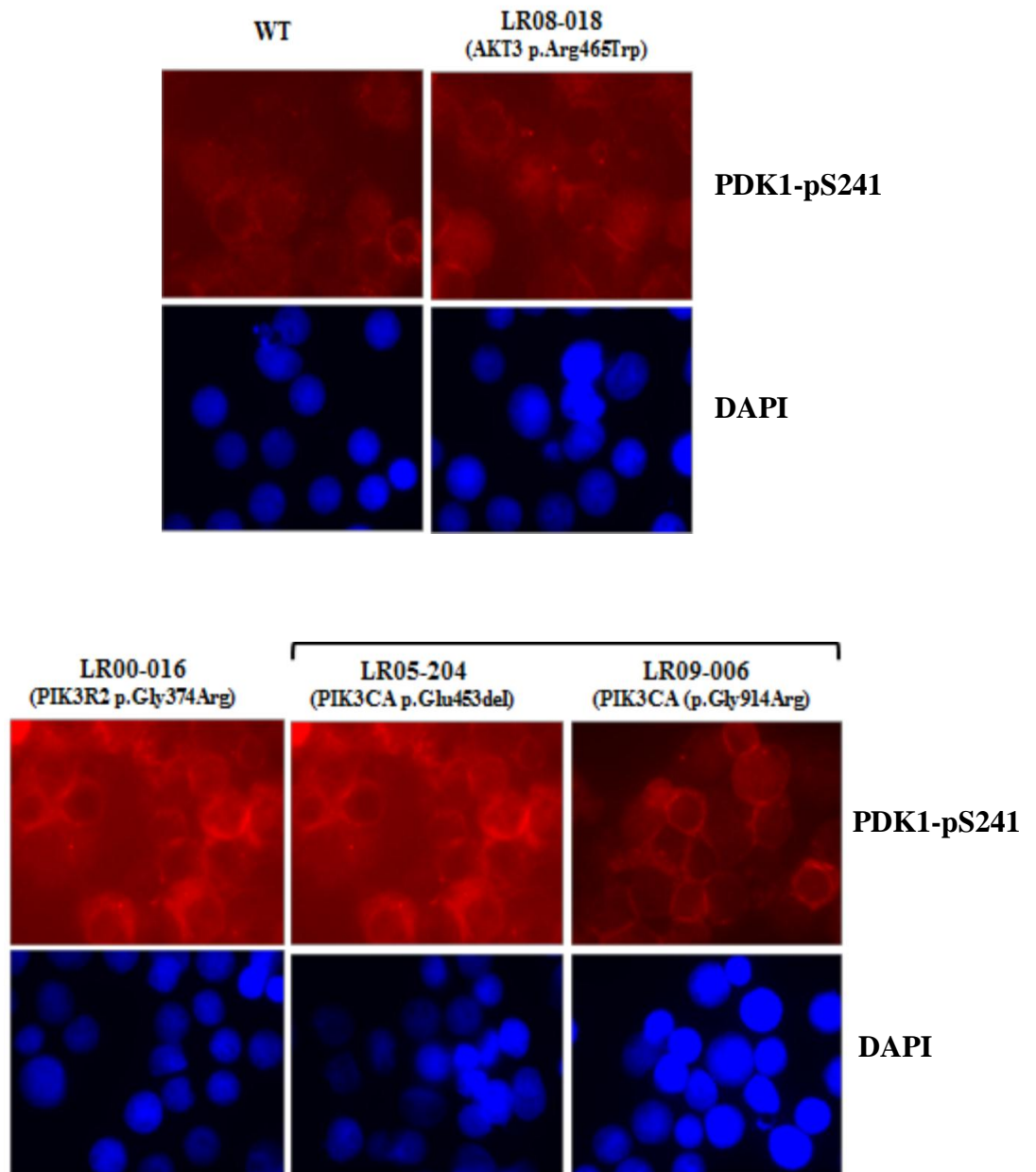


Figure 7.2.4. PIK3R2 and PIK3CA-mutated LBLs exhibit increased membrane-localized activated PDK1.

Indirect immunofluorescence (IF) staining of active PDK1 in exponentially growing lymphoblastoid cell lines using an anti-PDK1-p Ser241 antibody and a rhodamine-conjugated secondary antibody (red). Images were captured on the Zeiss AxioPlan platform using the same exposure times for each sample. Activated PDK1 is enriched at the cell membrane by binding to PIP3. Mutant lymphoblastoid cell lines derived from PI3K-mutated individuals LR00-016a1 (*PIK3R2* p.Gly373Arg), LR05-204 (*PIK3CA* p.Glu453del) and LR09-006 (*PIK3CA* p.Gly914Arg) all appear to exhibit increased membrane-localized endogenous PDK1-pSer241 compared to wild-type (WT) and *AKT3* mutant (LR08-018 p.Arg465Trp) cells. This is consistent with elevated PI3K activity in these cells. DAPI counterstain (blue) provides nuclear staining. 100X magnification.

7.2.3. *AKT3*, *PIK3R2* and *PIK3CA* mutant lymphoblastoid cells lines exhibit increased PI3K-AKT-mTOR pathway signaling: downstream from mTOR.

Chronic activation of the PI3K-AKT-mTOR network can be monitored via phosphorylation levels of downstream targets, such as S6 ribosomal protein and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (Figure 7.1.3).

Western blot analysis showed elevated endogenous levels of phosphorylated S6 protein and 4E-BP1 in all MCAP and MPPH mutant cell lines compared to control (WT) cells (Figure 7.2.5). Both *PIK3CA*, *PIK3R2* as well the *AKT3*-mutated patient LBLs therefore exhibit endogenously hyperactive/sustained signal via the PI3K-AKT-mTOR signaling network. Elevated S6 phosphorylation was also observed in a cell line derived from a Cowden's disease patient, which exhibits increased PI3K-mTOR signaling as a result of PTEN deficiency, a negative regulator of this pathway.

In order to determine if the increased mTOR-dependent signaling in MCAP and MPPH was a direct result of hyperactive PI3K, I treated MCAP and MPPH cells, mutant in *PIK3CA*, *PIK3R2* and *AKT3*, with a PI3K specific inhibitor. Treatment with PI-103, reduced S6 and TSC2 phosphorylation levels in control and mutant lines, however, the latter exhibited relative resistance to PI3K inhibition, which is consistent with elevated signaling through the pathway (Figure 7.2.6).

The precise structural mechanisms by which the different causative mutations result in increased PI3K activity (or AKT3) remain to be elucidated. However, the elevated levels of PIP₃ and increased phosphorylation of downstream mTOR signaling targets in the lymphoblastoid cell line derived from patient LR00-016a1 suggest that the *PIK3R2* p.Gly373Arg mutation results in increased PI3K lipid kinase activity. Importantly, the interaction between the helical domain of p110 and the first SH2 domain of p85, which is the location of the p.Gly373Arg mutation in *PIK3R2* (Figure 7.1.2), helps maintain the heterodimer in a low activity state. Although functional studies will be necessary to prove this pathogenic mechanism for p.Gly373Arg, this particular mutation is likely to result in increased PI3K activity by disrupting normal interaction with the catalytic subunit.

Altogether, these observations clearly show that the megalencephaly-associated mutations result in *increased* PI3K activity and PI3K-mTOR signaling, a

critical signal transduction pathway required for normal cell and organismal growth, representing a novel pathomechanism for these megalencephaly-related human disorders.

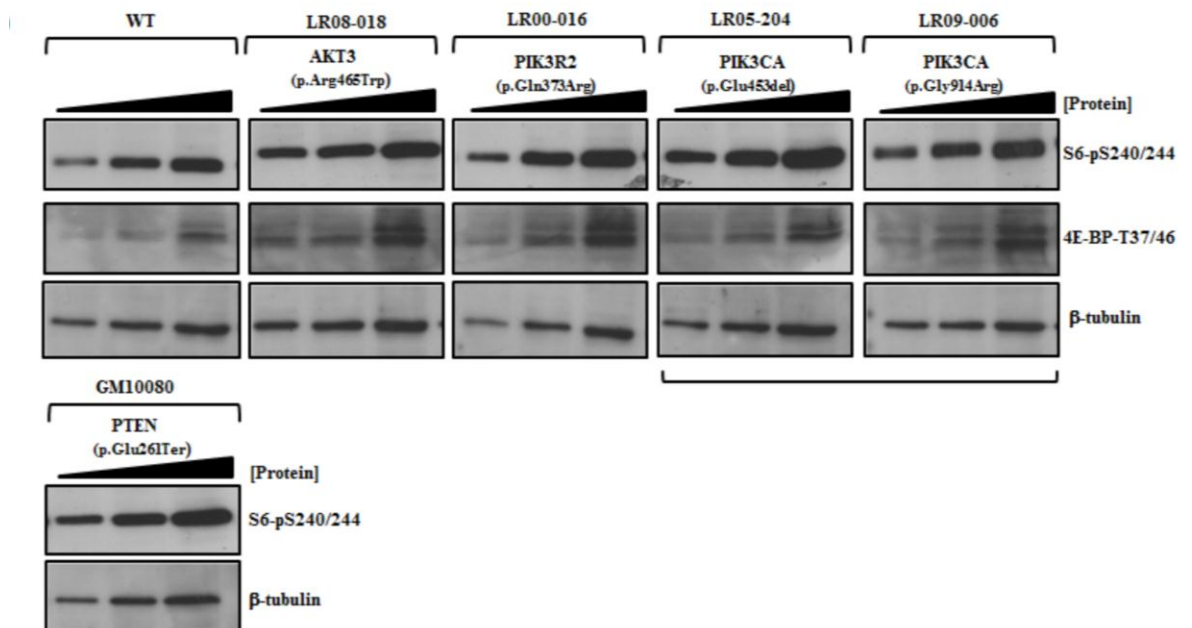


Figure 7.2.5. AKT3 and PI3K mutant lymphoblastoid cells lines exhibit elevated levels of phosphorylated S6 and 4E-BP1.

Western-blot analysis of phosphorylated S6 and 4E-BP1 in mutant and control (WT) LBLs. Endogenous levels of phosphorylated S6 and 4E-BP1 in differing amounts of whole cell extracts (5, 10 and 25µg) using phospho-specific antisera against pSer240/244 S6 and Thr37/47 4E-BP1. All mutant LBLs exhibited elevated levels of phosphorylated S6 and 4E-BP1 compared to WT. Elevated pSer240/244 S6 was also evident in GM10080, a lymphoblastoid cell line from a patient with Cowden' disease which served as a positive control. β-tubulin levels were used as a control for protein loading.

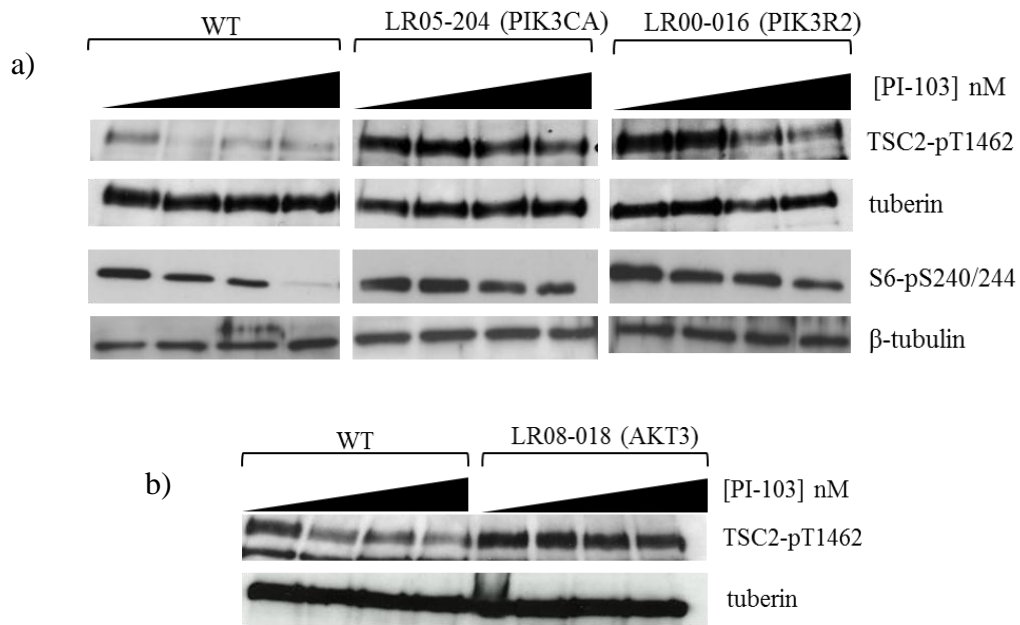


Figure 7.2.6. Sustained phosphorylation of PI3K-AKT downstream targets in MCAP/MPPH LBLs following treatment with the PI3K inhibitor, PI-103.

a) TSC2 and S6 phosphorylation was reduced to a greater extent in control cells (WT) when compared to those with *PIK3CA* (LR05-204 p.Glu453del) or *PIK3R2* (LR00-016 p.Gly373Arg) mutations upon exposure to increasing amounts of PI-103 (0, 50, 100, 200 nM) for 2 hours. **b)** Consistent with an *AKT3* activating mutation, treatment with PI3K inhibitor PI-103 did not inhibit TSC2 phosphorylation in the *AKT3*-mutated megalencephaly patient derived line, under the same conditions. These results further reflect elevated PI3K-mTOR signaling in MCAP and MPPH cell lines. β -tubulin and tuberin levels were used as a control for protein loading.

7.2.4. MCAP and MPPH patient derived LBLs exhibit inefficient ATR-dependent G2/M checkpoint.

Efficient DNA damage response is essential during normal brain development. MCAP and MPPH syndrome share a number of clinical features, including brain overgrowth, with disorders associated with *PTEN* loss of function. Interestingly, in Chapter 5, I showed that *PTEN*-deficient cells derived from Cowden's disease patients exhibit inefficient ATR-dependent checkpoint response, associated with increased PI3K signaling.

For this reason, I investigated aspects of the ATR-dependent DNA damage response in MCAP and MPPH patient derived LBLs, as a possible contributing mechanism underlying the megalencephaly in this disorder.

I found that MCAP and MPPH LBLs showed a moderately attenuated activation of the ATR-dependent G2/M cell cycle checkpoint in response to UV-induced damage (Figure 7.2.7) as efficiently as the control (WT) LBLs.

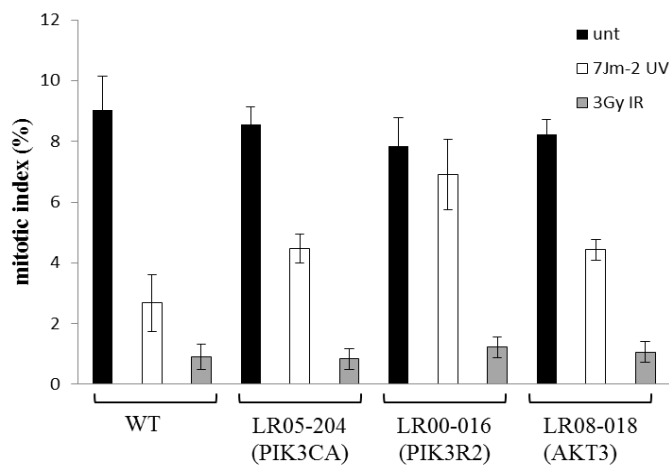


Figure 7.2.7. ATR-dependent G2/M checkpoint activation less efficient in MCAP and MPPH patient LBLs.

Control (WT), and megalencephaly patient LBLs were irradiated with either 7Jm⁻² or 3Gy IR, in the presence of colcemid, and the number of mitotic cells assessed 4hrs later. Patient derived cells failed to efficiently activate the ATR-dependent G2/M checkpoint arrest, compared to control cells. By contrast, the IR-induced (ATM dependent) checkpoint was efficiently activated in all patient derived LBLs under these conditions. Average + SD of 3 experiments.

This was particularly significant for the *PIK3R2*-mutated line. MCAP and MPPH mutant LBLs efficiently induced the IR-induced checkpoint arrest. This

suggests modest impairment specifically of the ATR-related (but not ATM) G2/M checkpoint in MCAP and MPPH. One possibility is that this could be the result of increased AKT activity in these cells, as increased AKT-dependent signaling has previously been known to overcome cell cycle checkpoints and induce mitosis in the presence of DNA damage (Kandel et al. 2002). Further work is required to interrogate this association. Nevertheless, my data point to a previously unappreciated association between attenuated ATR-dependent G2/M checkpoint proficiency with a megalencephaly disorder.

7.3. Summary

Megalencephaly-capillary malformation (MCAP) and Megalencephaly-polymicrogyria-polydactyly-hydrocephalus (MPPH) syndromes are sporadic overgrowth conditions. *De novo* germline or postzygotic mutations in three core components of the PI3K-AKT pathway were identified in MCAP and MPPH patients, including two mutations in *AKT3*, a recurrent mutation in *PIK3R2* and multiple mostly postzygotic mutations in *PIK3CA* (Figure 7.1.2).

Analysis of MCAP and MPPH patient LBLs demonstrated perturbed, *elevated* PI3K-mTOR signaling. This was confirmed by the elevated levels of PIP₃ and activated membrane associated PDK1 in *PIK3CA* and *PIK3R2*-mutated LBLs. PI3K and AKT3 mutant LBLs also exhibited increased phosphorylation of downstream mTOR signaling targets, S6 and 4E-BP1, as well as relative insensitivity to PI3K inhibition. Finally, MCAP and MPPH patient cells also showed somewhat inefficient G2/M cell cycle checkpoint arrest, associated with the ATR-dependent DNA damage response.

These results extend the list of human overgrowth syndromes associated with aberrant PI3K/AKT signaling and demonstrate that dysfunction of the PI3K/AKT pathway causes a constellation of developmental brain disorders including polymicrogyria, hydrocephalus, and cerebellar tonsillar ectopia including Chiari malformation with or without syringomyelia, as well as congenital vascular and limb malformations. The unanticipated association of inefficient ATR-dependent G2/M checkpoint control in this context may point to a dominant impact of spontaneously active AKT upon the DDR.

7.4. Discussion

In this chapter I designed and conducted functional characterization of lymphoblastoid lines derived from two related megalencephaly disorders, recently found to harbor mutations in core components of the PI3K-AKT pathway. Megalencephaly-capillary malformation (MCAP) and megalencephaly-polymicrogyria-polydactyly-hydrocephalus (MPPH) syndromes share a high number of clinical features, including primary megalencephaly, prenatal overgrowth, brain and body asymmetry, digital anomalies and connective tissue dysplasia. In addition, MCAP patients also exhibit cutaneous vascular malformations (Mirzaa et al. 2012; Mirzaa et al. 2004). The study of MCAP and MPPH patient derived cell lines aimed to further our knowledge of the role of PI3K-AKT signaling in brain, limb and vascular development.

The PI3Ks are a highly conserved family of signaling enzymes that regulate a wide range of processes including cell growth, proliferation, survival, migration, metabolism, angiogenesis, apoptosis and brain development (Vanhaesebroeck et al. 2001; Engelman, et al. 2006; Katso et al. 2001). The serine/threonine kinase AKT is the predominant downstream effector of PI3K signaling. *De novo* mutations in *AKT3* (a brain-predominant form of the AKT kinase), a recurrent mutation in *PIK3R2*, and multiple mostly postzygotic mutations in *PIK3CA* were identified in MCAP and MPPH patients (Rivière et al. 2012). *PIK3R2* and *PIK3CA* encode the p85 β regulatory subunit and the p110 α catalytic subunit of class IA PI3K, respectively.

To assess the impact of *AKT3*, *PIK3R2* and *PIK3CA* mutations on PI3K activity, I used immunostaining to compare phosphatidylinositol-4,5-trisphosphate (PIP₃) amounts in lymphoblastoid cell lines derived from four mutation carriers with megalencephaly to those in control and *PTEN*-mutant cells. Class IA PI3Ks convert phosphatidylinositol-4,5-bisphosphate (PIP₂) to PIP₃ in a reaction reversed by the tumor suppressor *PTEN* phosphatase (Figure 7.1.3). Strikingly, *PIK3CA* and *PIK3R2* mutated LBLs revealed significantly elevated levels of PIP₃ compared to WT levels. This phenotype strongly suggest elevated PI3K activity in these cells, and treatment with a specific PI3K inhibitor efficiently reduced PIP₃ levels in PI3K-mutated LBLs. Moreover, in MCAP and MPPH LBLs with *PIK3CA* and *PIK3R2* mutations there

was accumulation of active, phosphorylated PDK1 at the cell membranes, a PI3K dependent event (Bayascas 2010). Consistent with a mutation affecting a downstream mediator of PI3K and PDK1 signaling, I found no evidence for increased PI3K signaling in the *AKT3*-mutant line. Chronic activation of the PI3K-AKT-mTOR network can be monitored by examining the extent of phosphorylation of mTOR downstream targets, S6 ribosomal protein and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). Protein blot analysis showed higher amounts of phosphorylated S6 protein and 4E-BP1 in all MCAP and MPPH LBLs compared to controls, indicating mTOR hyperactivity in these cells. The TSC1-TSC2 complex is the main negative regulator of mTORC1, operating downstream of AKT (Huang et al. 2008). The AKT-dependent phosphorylation of TSC2 on threonine 1462 was elevated in MCAP and MPPH LBLs, indicating stronger inhibition of the TSC1-TSC2 complex. This results in release of TSC1-TSC2 complex inhibition over mTORC1, promoting activation of mTORC1 downstream targets. Mutant lines were also relatively resistant to PI3K inhibition. TSC2 and S6 phosphorylation upon exposure to increasing amounts of PI3K inhibitor, PI-103, was less reduced in MCAP and MPPH LBLs, in comparison to WT cells. These combined results suggest that the megalencephaly-associated mutations result in robust aberrant activation of PI3K-AKT-mTOR signal.

Substantial human and mouse model studies have shown that loss- and gain-of-function mutations in *AKT* isoforms lead to opposite phenotypes. The *Akt1* null mouse displays brain and somatic growth delay (Cho, et al. 2001), whereas an *AKT1* activating mutation was recently found to underlie Proteus syndrome (Lindhurst et al. 2011), an overgrowth disorder that shares clinical features with MCAP and MPPH. Whereas *AKT2* loss in the mouse and human causes insulin resistance and diabetes (Cho, Mu, et al. 2001; George et al. 2004), an activating *AKT2* mutation was associated with severe hypoglycemia and asymmetrical growth in humans (Hussain et al. 2011). The role of *AKT3* in controlling brain size has been well established. *Akt3* knockout in the mouse, and heterozygous *AKT3* loss in human have been associated with severe microcephaly (Ballif et al. 2012; Boland et al. 2007; Tschopp et al. 2005). On the contrary an activating mutation in the kinase domain of *Akt3* (p.Asp219Val) was shown to cause brain overgrowth and seizures in mice (Tokuda et al. 2011), a phenotype that resembles the clinical presentation of the

MCAP/MPPH *AKT3* mutation carriers. This data combined with my observations of higher S6 and 4E-BP1 phosphorylation in a lymphoblastoid cell line harboring the *AKT3* p.Arg465Trp alteration strongly suggests a gain-of-function mechanism resulting in enhanced AKT kinase activity for the two *AKT3* mutations in MCAP/MPPH. This is consistent with a recent study where somatic activating *AKT3* mutations were found in patients with hemimegalencephaly (brain hemisphere overgrowth), associated with severe intractable epilepsy (Poduri et al. 2012). Many MCAP and MPPH subjects had asymmetric brain enlargement, and several were diagnosed with hemimegalencephaly (Mirzaa et al. 2012). The findings presented in this chapter further emphasize the essential role of *AKT3* in regulating normal brain size, establish the association between brain overgrowth and increased AKT3-dependent signaling in two human disorders, and thus highlight the central but distinct functions of *AKT* isoforms in growth regulation and development.

Class IA PI3Ks are formed of a p110 catalytic and a p85 regulatory subunit. Each of these subunits has three isoforms encoded by three genes (p100 isoforms: *PIK3CA*, *PIK3CB* and *PIK3CD*; p85 isoforms: *PIK3R1*, *PIK3R2* and *PIK3R3*) (Engelman, Luo, and Cantley 2006). Mutations in five of these six genes (*PIK3CA*, *PIK3CB*, *PIK3CD*, *PIK3R1* and *PIK3R2*) have been observed in many human cancers (Cancer Genome Atlas Research Network 2008), but until now not in individuals with developmental disorders. The data presented in this chapter also shows that mutations in the PI3K p85 β (*PIK3R2*) regulatory and p110 α (*PIK3CA*) catalytic subunits resulting in aberrant activation of PI3K, are a common cause of megalencephaly.

A small number of malignancies and benign tumors have been observed in some patients with MCAP and MPPH syndromes. Regarding MCAP, the literature documents two cases of Wilms' tumor (Lapunzina et al. 2004; D. R. Wright et al. 2009), one of whom also experienced cutaneous dermatomyofibroma and lipomas of the foot. A patient from the original series by Moore *et al.* died of leukemia at age 18 years (Moore et al. 1997). A single MPPH patient has been reported with a medulloblastoma (Osterling et al. 2011). Numerous benign growths have also been seen in MCAP, of which vascular growths are the most common (Mirzaa et al. 2012; Moore et al. 1997; Martínez-Glez et al. 2010; Clayton-Smith et al. 1997). Furthermore, in one patient a large tufted angioma of the shoulder led to Kasabach-

Merritt syndrome during early infancy (Wright et al. 2009). The large cohort reported by Mirzaa *et al.* also described one patient with multiple lipomas but no other growths (Mirzaa et al. 2012). Finally, two patients have been described with pediatric meningioma, which are extremely rare tumors in children (Moore et al. 1997; Conway et al. 2007).

The regulatory p85 subunits are known to stabilize the catalytic subunits, influence their localization and binding partners, and maintain them in a low activity state (Miled et al. 2007; Chuan-Hsiang Huang et al. 2007). Importantly, the interaction between the helical domain of p110 and the first SH2 domain of p85, helps maintain the heterodimer in a low activity state. The p.Gly373Arg mutation in *PIK3R2* found in MPPH is located in this region, and it has been found in cancer, suggesting that this particular mutation may result in increased PI3K activity by disrupting normal interaction with the catalytic subunit. Somatic activating mutations in *PIK3CA* are frequently observed in approximately 30% of colorectal, breast and endometrial cancers, and at a lower frequency in other tumor types such as glioblastoma multiforme and skin cancer (Samuels et al. 2004).

Presently, new mosaic gain-of-function mutations in *PIK3CA* have also been implicated in other distinct overgrowth conditions. Mutations in *PIK3CA*, associated with enhanced activation of the PI3K-AKT-mTOR pathway, were identified in hemimegalencephaly patients (also mutations in *mTOR* and *AKT3*) (J. H. Lee et al. 2012), mosaic overgrowth disorder with fibroadipose hyperplasia (Lindhurst et al. 2012), congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies (CLOVES) syndrome (Kurek et al. 2012) and macrodactyly (Rios et al. 2012). Phenotypic differences observed among all these overgrowth disorders could be associated with the timing and occurrence of the different causative mutations during embryogenesis, as well as the *degree* of PI3K-dependent activation of common downstream pathways.

MCAP and MPPH also share clinical features with a spectrum of overgrowth disorders caused by germline loss of function mutations in *PTEN*, a negative regulator of PI3K activity. These include Cowden disease, Bannayan-Riley-Ruvalcaba syndrome and autism with severe megalencephaly (Butler et al. 2005;

Liaw et al. 1997; Marsh et al. 1998). Autism spectrum disorder can also be part of the neurocognitive phenotype of MCAP/MPPH subjects.

Additionally, the PI3K/AKT pathway is a major downstream effector of RAS (Figure 7.2.8), and the interaction of RAS with class I PI3K isoforms is well documented (Castellano et al. 2010), which may explain the significant overlap between MCAP/MPPH and the group of disorders globally known as RASopathies.

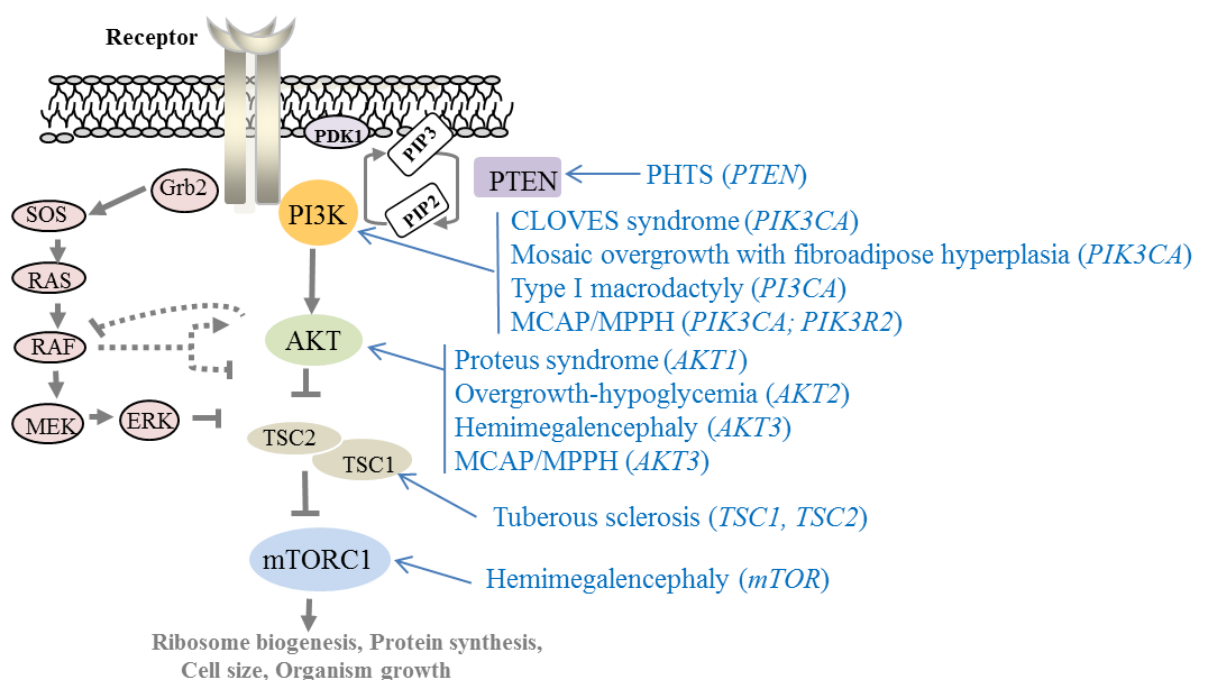


Figure 7.4.1 PI3K-AKT signaling pathway overview and the known overgrowth syndromes associated with mutations in components of this pathway.

Activated receptor-tyrosine kinase leads to activation of PI3K family members, which in turn convert phosphatidylinositol-3,4-bisphosphate (PIP₂) to the -3,4,5-triphosphate (PIP₃), a process that is antagonized by the PTEN phosphatase. At the plasma membrane, PIP₃ facilitates recruitment and phosphorylation of PDK1. PDK1 then promotes the activating phosphorylation of AKT at Thr308. Activated AKT inhibits the main negative regulator of mTORC1, the TSC1-TSC2 complex, resulting in mTORC1 activation. PHTS: PTEN Hamartoma Tumor Syndrome; CLOVES: Congenital Lipomatous Overgrowth Vascular malformation Epidermal nevi, Skeletal/spinal anomalies.

Finally, the MCAP and MPPH patient LBLs analyzed here also exhibit an inefficient G2/M cell cycle checkpoint arrest, associated with the ATR-dependent (but not ATM) DNA damage response. Interestingly, *PTEN*-deficient cells have also been associated with defects specifically in the ATR-dependent G2/M checkpoint (see Chapter 5), and Cowden's disease and other disorders associated with *PTEN* deficiency, share clinical features with MCAP and MPPH, including brain overgrowth. It is possible that the checkpoint defect observed in these particular disorders could be associated with increased AKT-dependent signaling, which has been known to surpass cell cycle checkpoints and induce mitosis in the presence of types of DNA damage (Kandel et al. 2002). This data may further support the importance of an efficient DNA damage response during normal brain development.

In conclusion, the data presented in this chapter extends the list of human overgrowth syndromes associated with aberrant PI3K/AKT signaling (Figure 7.4.1) and demonstrate that dysfunction of PI3K/AKT pathway signaling causes a constellation of brain, vascular and limb malformations. These findings combined with the development of inhibitors for PI3K, AKT or downstream targets such as mTORC1 (rapamycin treatment, for example, has been shown to benefit children with somatic *PTEN* deficiency (Marsh et al. 2008) could provide new therapeutic opportunities for megalencephaly as well as other developmental syndromes.

Chapter Eight

Results VI: Characterization of aspects of mTOR pathway function in cells from a Genomic Disorder caused by copy-number variation of 1q21.1

8.1 Introduction

Copy Number Variations (CNVs) are structural genomic modifications that result in altered number of copies of one or more regions of the DNA. CNVs generally encompass large regions (>1Kb), and are an important contributor to normal genomic variation (Stankiewicz and Lupski 2010). Locus-specific rates of genomic CNVs occur at several orders of magnitude higher than SNPs (10^{-6} to 10^{-4} , versus 10^{-8}). Therefore, CNVs are likely a more frequent cause of human congenital disorder when compared to SNPs (Lupski et al. 2007). Region-specific low copy repeats (LCR) are susceptible for non-allelic homologous recombination (NAHR), which can result in modifications including deletions, duplications or complex rearrangements (inversions, translocations). Such structural alterations in the human genome are commonly found in cancers (Stankiewicz and Lupski 2010; Colnaghi et al. 2011). Additionally, CNVs can also result in congenital Genomic Disorders, often accompanied by complex phenotypic traits (Colnaghi et al. 2011).

In this chapter I set out to understand the impact of CNV on gene expression and function of a particular gene, using cell lines from clinically well characterized patients with duplications or deletions in the 1q21.1 chromosomal region.

Copy number changes of 1q21.1 (deletion, OMIM 612474; duplication, OMIM 612475) are associated with variable phenotypes. These include intellectual disability and/or autism (Brunetti-Pierri et al. 2008), schizophrenia (Stefansson et al. 2008; Shtivelman et al. 2002; Christiansen et al. 2004; Xu et al. 2010), congenital heart anomalies (Kandel et al. 2002; Mefford et al. 2008), dysmorphic features, including microcephaly/macrocephaly (Xu et al. 2010; Brunetti-Pierri et al. 2008) (Figure 8.1.1). My interest was to try and gain some understanding into why microcephaly was associated with 1q21.1 deletion and macrocephaly with 1q21.1 duplication.

The 1q21.1 critical region spans approximately 1.35 Mb (from 145 to 146.35 Mb) and includes at least 12 genes (Mefford et al. 2008) (Figure 8.1.2). Functional consequences of genes integral to CNVs in cells/tissues from carriers are rarely studied, due to unavailability of appropriate human tissues and the rarity of patients with individual CNVs (O'Driscoll et al. 2007). For these reasons, the underlying genetics basis associating these phenotypes with 1q21.1 copy number variant (CNV) remains largely unknown.



Figure 8.1.1. Facial appearance of individuals with the 1q21.1 microdeletion/microduplication.

Microcephaly and certain facial dysmorphic features such as frontal bossing, deep-set eyes and bulbous nose seem to be common in individuals with 1q21.1 microdeletion (top row); and macrocephaly, frontal bossing and hypertelorism in individuals with 1q21.1 microduplication (bottom row). Developmental delay and/or learning disabilities are reported in most cases, as well as behavioral abnormalities, including attention deficit hyperactivity disorder (ADHD), autism, anxiety/depression, antisocial behavior, aggression and even hallucinations (adapted from Brunetti-Pierri et al.2008).

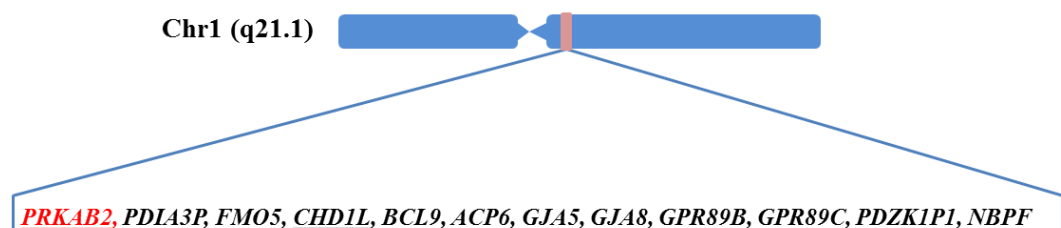


Figure 8.1.2. Genes included in the 1q21.1 CNV critical region. Core genes seen in all subjects with a 1q21.1 CNV. Two of the genes that ranked highest in the expression/1q21.1 copy number correlation were studied (underlined). I investigated aspects of *PRKAB2* function (in red), which encodes the $\beta 2$ isoform of AMPK, in patient cells. (Harvard et al., 2011)

Our collaborator, Evica Rajcan-Separovic's group (Vancouver, Canada) performed whole transcriptome expression analysis in subjects with 1q21.1 deletions and duplications and ranked the top genes whose expression correlated highest with the 1q1.1 copy number (Harvard et al. 2011). The impact of the 1q21.1 CNV on gene expression and function of two selected top genes within that region was then evaluated.

CHD1L, the gene which ranked first in the expression/copy number correlation, has been implicated in chromatin remodeling and relaxation as well as DNA repair (Deng et al. 2009; Ahel et al. 2009). A novel role for *CHD1L* in decatenation (chromatin untangling) checkpoint activation was identified, which was suspected based on its known chromatin remodeling function, and the defective Topo II decatenation checkpoint demonstrated in both the 1q21.1 deletion and duplication containing patient cell lines (Harvard et al. 2011).

PRKAB2, which encodes one of the AMP-activated protein kinase (AMPK) isoforms, is also found in the 1q21.1 critical region, and ranked 10th in the correlation between expression/copy number analysis (Harvard et al. 2011).

AMPK is a highly conserved heterotrimeric kinase that plays a major role in sensing and responding to the cell's energy status (Kahn et al. 2005). AMPK is activated by the reduction in ATP levels, and the correspondingly reciprocal increase in AMP or ADP (Figure 8.1.3), which accompanies a variety of physiological processes, such as low nutrient availability, prolonged exercise or pharmacological agents. Conformational changes involving the autoinhibitory domain (AID) of AMPK, both promote and protect phosphorylation of the key residue Thr172 on the AMPK- α subunit, essential for AMPK activation (Young et al. 2009). The LKB1 (liver kinase B1) kinase is an important mediator of AMPK- α Thr172 phosphorylation. Additionally, AMPK can be activated in response to calcium influx, involving CaMKK (Calcium/calmodulin-dependent protein kinase kinase) (Kahn et al. 2005). AMPK controls a number of metabolic processes, including glucose uptake, fatty acid and glucose metabolism and mitochondria biogenesis. Moreover, the AMPK signal transduction pathway interacts with the PI3K/AKT/mTOR network to regulate cell growth, protein translation and autophagy in response to cellular stress (Garelick et al. 2011) (Figure 8.1.4).

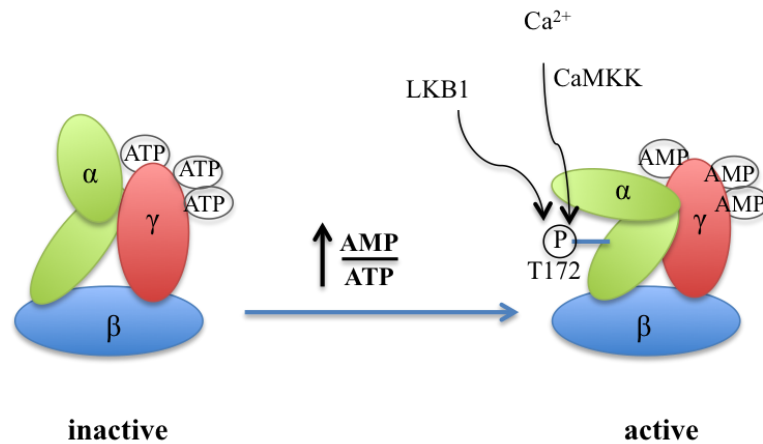


Figure 8.1.3. AMPK activation model.

The AMPK heterotrimeric complex is formed by the α catalytic subunit (green), which includes the kinase domain containing the key Thr172 residue and an autoinhibitory domain; the β subunit (blue) containing a glycogen binding domain and binding sites for both the α and γ subunits; and the γ subunit, which contains the nucleotide binding sites. When the AMP/ATP ratio increases, more AMP is bound to the γ subunit and the auto-inhibitory subunit releases from the hinge of the kinase domain, which then assumes its active more closed configuration. Access of protein phosphatases (PP2C) to Thr172 is limited, favoring the action of upstream kinases (LKB1 and CaMKK) resulting in a high phosphorylation state. (Adapted from Young et al. 2009).

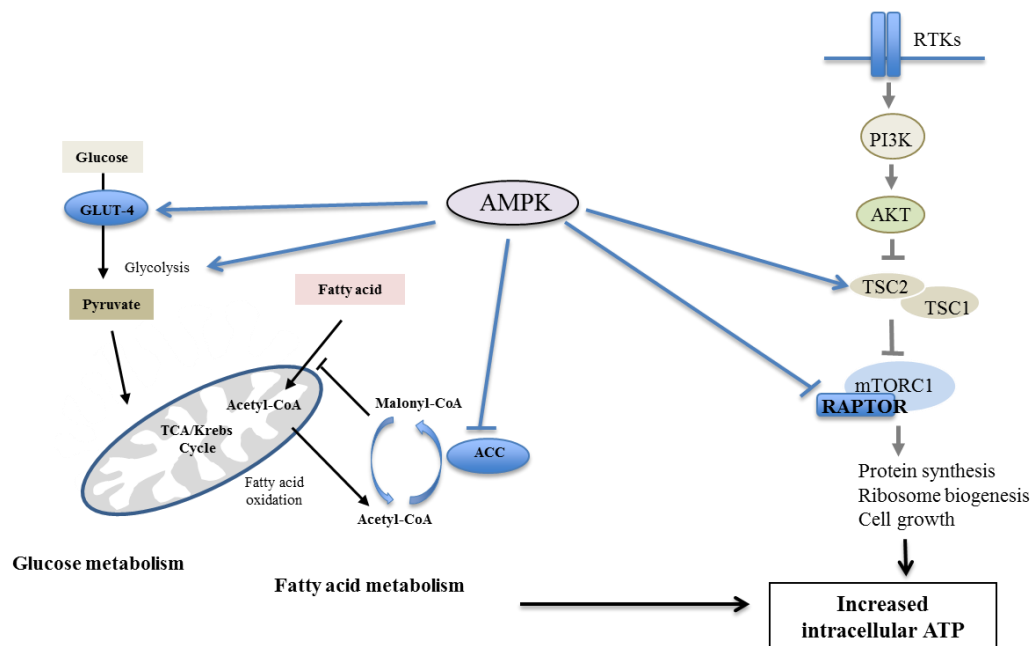


Figure 8.1.4. Activated AMPK directly phosphorylates substrates involved in cell metabolism and growth.

Phosphorylation and consequent inactivation of ACC by AMPK regulates fatty acid metabolism by reducing malonyl-CoA inhibitory effect on mitochondrial fatty acid oxidation. AMPK activation also promotes glucose metabolism and has an inhibitory effect on mTOR pathway via TSC2 activation and phosphorylation of RAPTOR, suppressing protein synthesis when cellular energy and nutrients become limiting.

PRKAB2 encodes the $\beta 2$ isoform of the AMPK (Young 2009). Since AMPK can influence mTOR function and because the mTOR pathway plays such a fundamental role in normal tissue and neuronal development and function, I set out to establish whether AMPK function was affected by *PRKAB2* CNV in cells from 1q21.1 CNV individuals. Importantly, I utilized cells bearing 1q21.1 deletion and duplication.

8.2 Results

8.2.1. AMPK- β 2 and AMPK- β 1 expression levels in patient-derived LBLs with 1q21.1 CNV

AMP-activated protein kinase (AMPK) is a heterotrimeric protein complex formed of three subunits: a catalytic α -subunit, a regulatory β -subunit and an ATP/ADP-binding γ -subunit. These subunits can exist in different isoforms (α 1, α 2, β 1, β 2, γ 1, γ 2, γ 3) (Young et al. 2009). *PRKAB2*, located within the 1q21.1 CNV region, encodes the β 2 isoform of the AMPK.

AMPK- β 2 expression, the protein product of *PRKAB2*, was reduced in the cell line with the 1q21.1 deletion (Del) and increased in the cell line with the 1q21.1 duplication (Dup), by comparison to a control unaffected (WT) cell line (Figure 8.2.1 a,b). By contrast, expression of the closely related β 1 AMPK subunit was not altered in the Dup and Del lines, compared to WT levels (Figure 8.2.1c). Importantly, *PRKAB1*, the gene that codes for AMPK- β 1, is located on chromosome 12q24.1, not within the 1q21.1 CNV. This data shows for the first time that protein levels of AMPK- β 2 are altered in accordance with the 1q21.1 copy number state and expression levels of *PRKAB2* (Harvard et al. 2011). It was therefore plausible that AMPK function could be affected in 1q21.1 CNV cell lines as a result of altered expression of the AMPK- β 2 subunit.

8.2.2. AICAR-induced activation of AMPK kinase in patient-derived LBLs with 1q21.1 CNV

AMPK is a key sensor and regulator of energy homeostasis in the cell, which is activated by an elevated AMP/ATP ratio. The nucleoside analogue AICAR (N1-(β -D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide) is a cell permeable compound that mimics the effects of AMP on the allosteric activation of AMPK. To investigate the effect of increased and decreased expression of AMPK- β 2 on AMPK activity, I treated patient-derived LBLs with AICAR and monitored activation of AMPK on threonine-172 (p-T172-AMPK α). This is an essential modification, required for and diagnostic of AMPK activity.

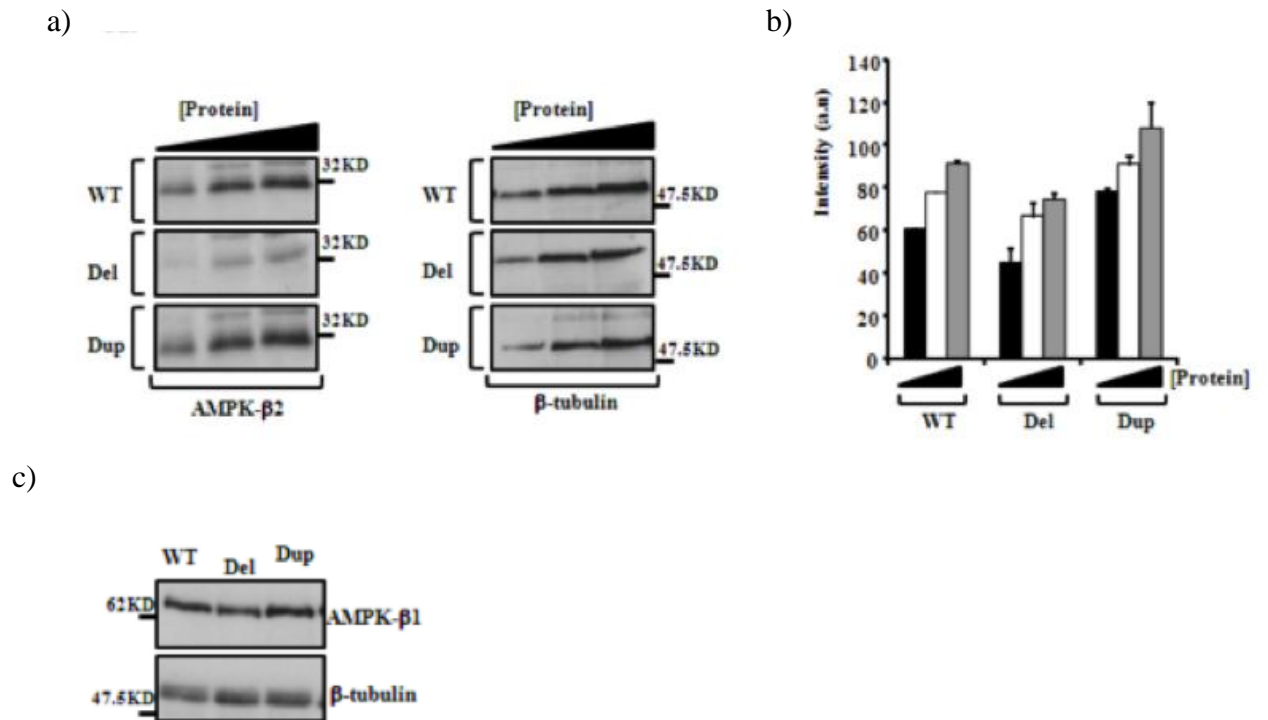


Figure 8.2.1. AMPK- β 2 and AMPK- β 1 expression levels in patient-derived LBLs with 1q21.1 CNV.

a) Titrated whole cell extracts were blotted for AMPK- β 2 (encoded by *PRKAB2*) in wild-type (WT), Del and Dup containing LBLs. The blots were re-probed with anti- β -tubulin. **b)** Densitometric quantification of AMPK- β 2 expression from titrated extracts, going from low (black bar), intermediate (white) to higher (grey) amounts of protein, normalized to β -tubulin loading, from three separate determinations (a.u. arbitrary units). $p = 0.01$ for Del and $p < 0.005$ for Dup LBCs compared to WT. **c)** AMPK subunit AMPK- β 1, encoded by the *PRKAB1* gene on chromosome 12q24.1, shows equivalent expression in the wild-type (WT), Del and Dup containing LBCs. β -tubulin was used to confirm equal loading.

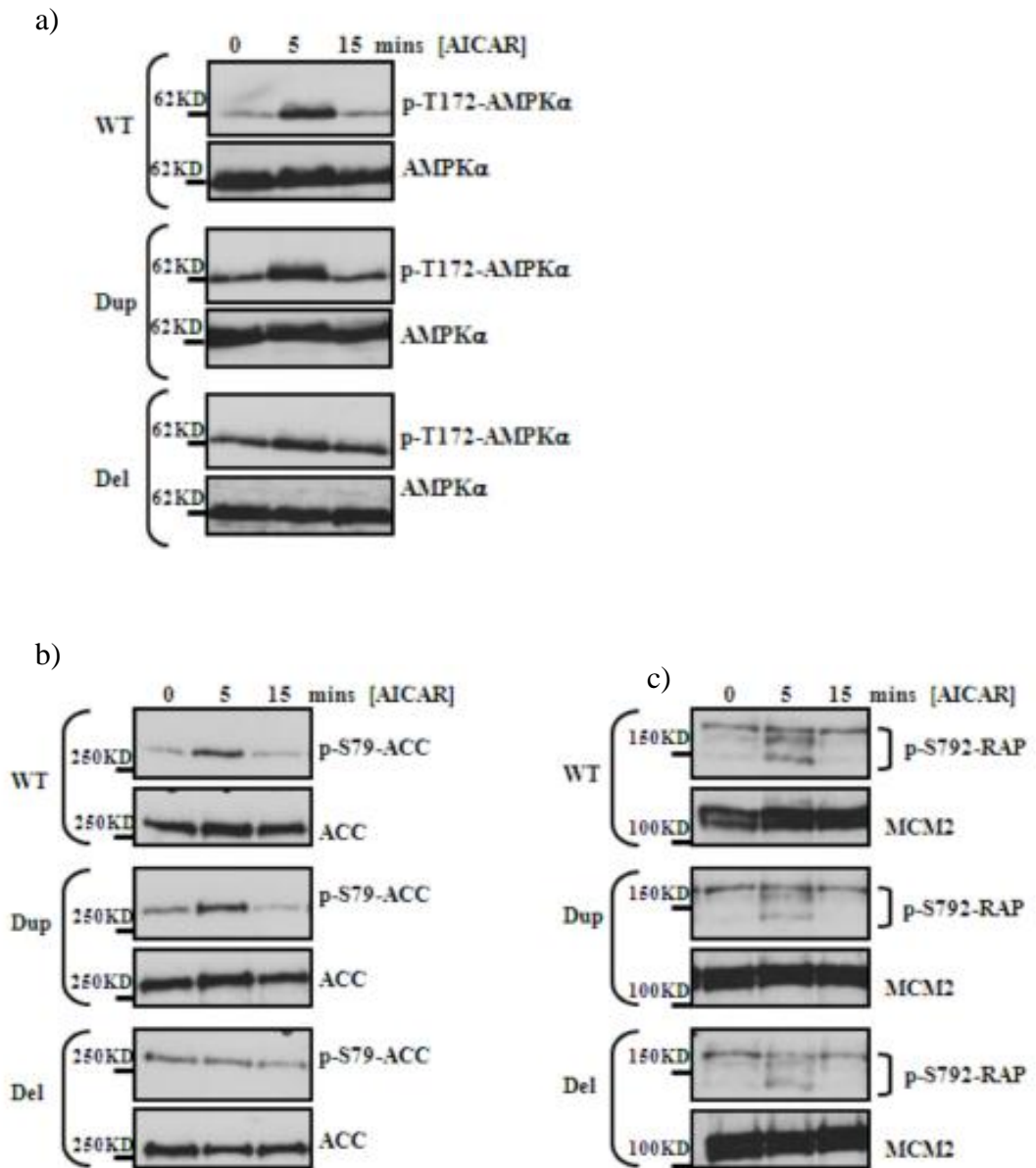


Figure 8.2.2. AICAR-induced activation of AMPK kinase in patient LBLs with 1q21.1 CNV.

a) AICAR-induced (2mM) activation of AMPK kinase was monitored using phosphorylation of the AMPK α subunit on threonine 172 (p-T172-AMPK α). Dup and Del containing LBLs exhibited higher levels of p-T172-AMPK α at the 0 time (untreated), relative to wild-type (WT). Only the 1q21.1 Del containing LBLs appeared to be unresponsive to AICAR-treatment. Blots were re-probed with for native AMPK α to confirm loading. **b)** AICAR-induced (2 mM) activation of AMPK was evaluated by monitoring phosphorylation of the AMPK substrate Acetyl-CoA Carboxylase on serine 79 (p-S79-ACC). Similar to p-T172-AMPK α , the Del containing LBCs appear unresponsive to the AICAR treatment. Blots re-probed for native ACC to confirm loading. **c)** AICAR-induced activation of AMPK was also evaluated by phosphorylation of the AMPK substrate RAPTOR on serine 792 (p-S792-RAP) under identical conditions. Again, Del containing LBCs appeared somewhat unresponsive to AICAR. Blots were re-probed for MCM2 to confirm loading.

Interestingly, the 1q21.1 Dup and 1q21.1 Del containing cell lines exhibited slightly elevated basal levels of p-T172-AMPK α prior to treatment with AICAR, compared to control (WT) cells (Figure 8.2.2a). Within 5 minutes following AICAR treatment, increased phosphorylation of p-T172-AMPK α was induced in wild-type LBLs. Elevated AICAR-induced p-T172-AMPK α was also detected in the 1q21.1 Dup containing cells. In comparison, the change in p-T172-AMPK α after 5 minutes AICAR induction was less apparent in the 1q21.1 Del containing line, and the activity remained constant at 15 minutes. In contrast, the AICAR-induced p-T172-AMPK α activity of the WT and 1q21.1 Dup LBL was reduced after 15 minutes. This data suggests that reduced AMPK- β 2 level is associated with somewhat unresponsive AMPK activation, while the 1q21.1 duplication-containing LBL showed similar pattern of responsiveness to WT cells under these conditions.

To investigate the significance of these findings, I analyzed the AMPK-mediated phosphorylation of two of its best known substrates, Acetyl-CoA Carboxylase (ACC) and RAPTOR. ACC is a key mediator of fatty acid (FA) synthesis. AMPK-induced phosphorylation of ACC on serine-79 (p-S79-ACC) inhibits ACC enzymatic activity thereby limiting FA synthesis under energy limiting conditions (i.e. high [AMP] and low [ATP]) (B. B. Kahn et al. 2005). Consistent with the findings with p-T172-AMPK α , I found efficient induction of p-S79-ACC in control (WT) and 1q21.1 Dup LBLs within 5 minutes of AICAR treatment (Figure 8.2.2b). Significantly, the LBLs with 1q21.1 Del failed to exhibit significant levels of p-S79-ACC under the same conditions. This data supports the observation of sub-optimal AMPK activity in the 1q21.1 deletion-containing cell line.

RAPTOR is an important regulatory component of the mTOR containing complex 1 (mTORC1), required for optimal mTOR kinase activity (D.-H. Kim et al. 2002). Under conditions when cellular energy is limiting, AMPK mediates phosphorylation of RAPTOR on serine-792 (p-S792-RAP), promoting inhibition of mTORC1 thereby limiting protein synthesis and inducing cell cycle arrest. Consistent with sub-optimal AMPK activity in the 1q21.1 Del containing LBLs, I found reduced AICAR-induced p-S792-RAPTOR in these cells in contrast to the 1q21.1 Dup containing line and the WT control cells (Figure 8.2.2c). These results suggest that reduced expression of the AMPK- β 2 subunit is associated with impaired AICAR-induced activation of AMPK. In contrast, duplication of *PRKAB2* did not negatively impact on AMPK activity under these conditions.

8.2.3. Characterization of mitochondria activity in 1q21.1 CNV LBLs

Activation of AMPK can promote mitochondrial biogenesis and function in order to increase cellular energy levels. AICAR stimulation of AMPK activity in rat quadriceps has been shown to result in increased expression of mitochondrial enzyme cytochrome c as well as the Glucose transporter type 4 (GLUT-4) (Leick et al. 2010). Inhibition of acetyl-CoA carboxylase (ACC), a major AMPK substrate, promotes mitochondrial fatty acid oxidation (Kahn et al. 2005). The peroxisome proliferator-activated receptor gamma coactivator-1- α (PGC-1 α) is an important regulator of mitochondrial biogenesis which is activated by AMPK (Jäger et al. 2007; Zong et al. 2002). Additionally, mitochondria function is important for synaptic plasticity and its deregulation has been associated with numerous neuropathologies. Consequently, I investigated whether the neurological phenotype of 1q21.1 CNV carriers could be associated with abnormal mitochondrial function, at least for the Del LBL exhibiting compromised AMPK function.

Interestingly, reduced mitochondrial content has been observed in AMPK β 2 deficiency mice (although more significantly so after β 1 or combined β 1 β 2 knockdown) associated with reduced mitochondrial citrate synthase and cytochrome c oxidase activity (O'Neill et al. 2011).

I used mitochondrion-specific fluorescent dyes (MitoTracker, Invitrogen) to characterize mitochondria function/status in LBLs with 1q21.1 Dup or Del, using flow cytometry. MitoTracker Red probe accumulation in the mitochondria of live cells is indicative of the status of the mitochondrial transmembrane electrical potential. MitoSox dye selectively detects superoxide in the mitochondria, indicative of mitochondria-derived ROS generation as a by-product of oxidative phosphorylation. MitoTracker Green FM Dye is used to determine mitochondrial mass (i.e. amount of mitochondria within the cells). The intensity of MitoTracker Red and MitoSox fluorescence in both 1q21.1 Dup and 1q21.1 Del LBLs was comparable to control (WT) LBLs, suggesting normal mitochondria activity. This data does not suggest impaired mitochondria function in 1q21.1 CNV LBLs. However, the modest increased relative MitoGreen fluorescence in 1q21.1 Dup LBL, compared to WT levels, suggests a potential increase in total mitochondria mass in these cells, although the significance of this is yet not clear. However, importantly,

1q21.1 deletion does not seem to negatively impact on mitochondrial mass or function.

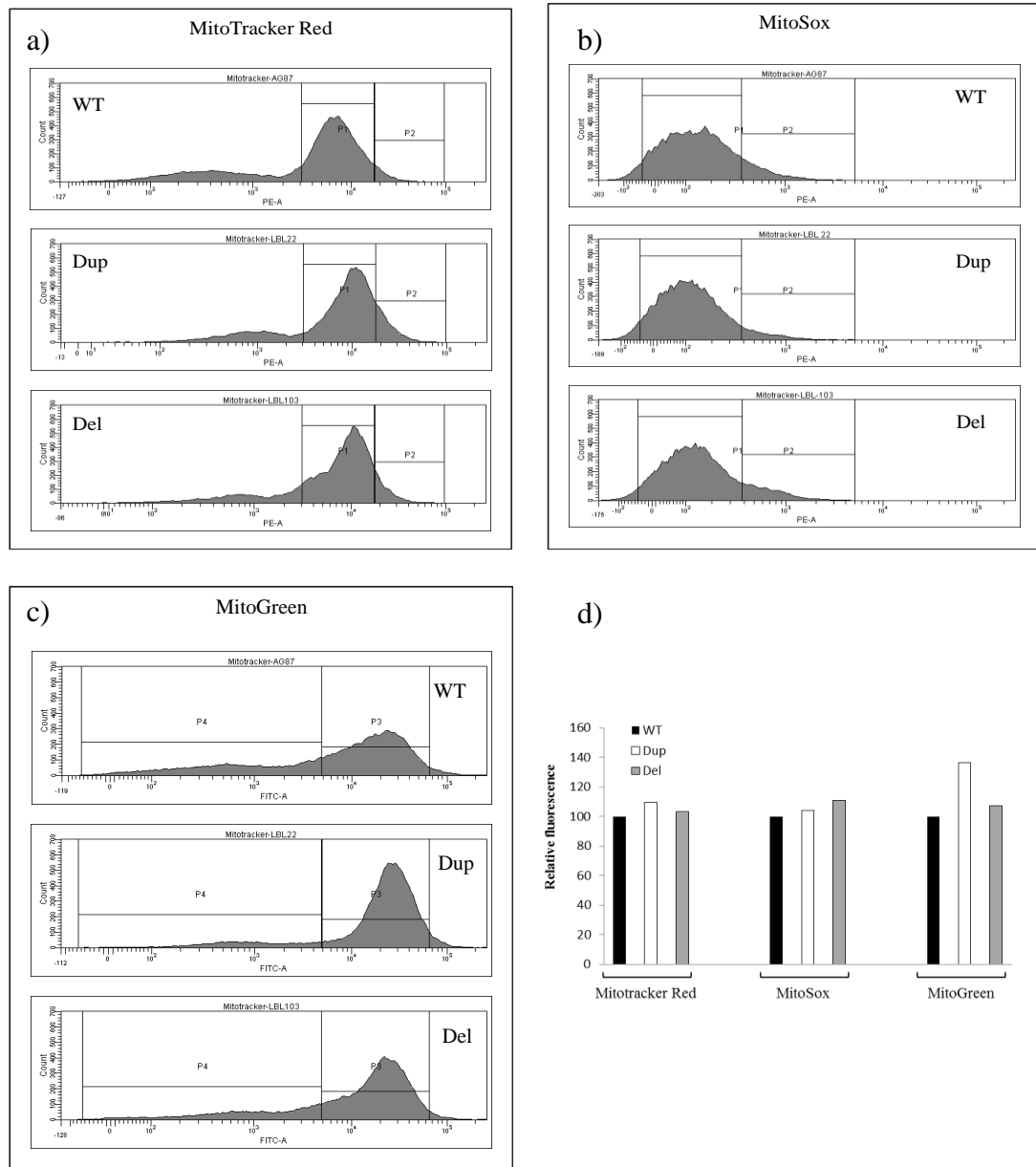


Figure 8.2.3. Characterization of mitochondria function in 1q21.1 Dup and Del LBLs.

WT, 1q21.1 Dup and 1q21.1 Del LBLs were stained with different mitochondrion selective probes (MitoTracker®, Invitrogen). a) MitoTracker Red dye, which is sensitive to mitochondrial membrane potential; b) MitoSox, indicates intracellular superoxide formation and c) MitoGreen, associated with total mitochondria mass, and the fluorescence was determined by FACS analysis. d) relative fluorescence intensity for the different mitochondria staining dyes in control, 1q21.1 Dup and 1q21.1 Del LBLs indicates normal mitochondrial function in these CNV carrier lines.

8.3 Summary

1q21.1 Copy Number Variant (CNV) is associated with a highly variable phenotype. 1q21.1 CNV carriers exhibit learning problems/ intellectual disability (ID) of various degrees, congenital anomalies, dysmorphic features, making the clinical significance of this CNV difficult to evaluate.

Here I describe the consequences of the 1q21.1 CNV on gene expression and function of a selected candidate gene within 1q21.1 (*PRKAB2*) using cell lines from clinically well described subjects.

I show that the 1q21.1 CNV can not only cause changes in the expression of one of the genes within the CNV, but also results in changes in their known function, as exemplified by impaired AMPK activation in the 1q21.1 Del LBLs. Haploinsufficiency of *PRKAB2* results in reduced expression of AMPK- β 2, which is associated with impaired AICAR-induced AMPK activation. In contrast, duplication of *PRKAB2* did not negatively impact on AMPK activity under these conditions. Preliminary data suggests mitochondria function is not impaired in 1q21.1 CNV carriers. These results support the use of patient lymphoblasts for dissecting the functional consequences of genes integral to CNVs in carrier cell lines, ultimately increasing understanding of biological processes which may contribute to the clinical phenotype.

8.4 Discussion

Copy Number Variation of chromosomal region 1q21.1 is associated with a highly variable phenotype (Mefford et al. 2008). Learning difficulties and intellectual disability (ID) of various degrees are one of the most commonly observed features of 1q21.1 CNV carriers. Congenital heart anomalies, developmental delay, dysmorphic features, schizophrenia, behavioral abnormalities or even a normal phenotype, have also been associated with 1q21 CNV (Christiansen et al. 2004; Brunet et al. 2009; Stefansson et al. 2008; Mefford et al. 2008). Additionally, microcephaly and macrocephaly have been observed in subjects carrying microdeletions or microduplications of this region, respectively (Brunetti-Pierri et al. 2008). The broad clinical presentation of 1q21.1 CNV carriers means that the biological consequences of 1q21.1 CNV have been difficult to establish.

Recently, whole transcriptome expression analysis was performed in carriers of 1q21.1 deletion and 1q21.1 duplication (Harvard et al. 2008). This study allowed the identification of a core group of genes in the critical region, which could be ranked based on correlation of expression and 1q21.1 copy number change.

CHD1L was the gene that ranked highest in the expression/ 1q21.1 copy number correlation. *CHD1L/ALC1* is an enzyme involved in chromatin modification and the DNA damage response. Functional studies in 1q21.1 CNV LBLs established a new role for *CHD1L* in chromatin untangling (decatenation), whereby 1q21.1 Del and Dup containing patient cell lines exhibited a defective Topo II decatenation checkpoint (Harvard et al. 2008). Similar decatenation checkpoint defects have been observed in Werner syndrome (Franchitto et al. 2003).

As part of that study (manuscript included in the Publications section), in this chapter I characterized aspects of AMPK functionality in cell lines from 1q21.1 CNV carriers (deletion and duplication), whose critical region includes *PRKAB2*, the gene that codes for the β subunit of AMPK. *PRKAB2* ranked 10th in the expression/1q21.1 copy number correlation (Harvard et al. 2008).

AMPK is a key regulator of cellular response to a large number of external stimuli, which modulates energy levels at the cellular and organism level. I found deregulation of AMPK- β 2 expression in the 1q21.1 deletion and duplication carriers. Changes in levels of AMPK- β 2 protein were observed, in keeping with the 1q21.1

copy number state and expression level of the *PRKAB2* gene. Phosphorylation of AMPK- α on Thr172 is an essential modification required for AMPK activation (Young et al. 2009). Both 1q21.1 Del and Dup containing lines exhibit different basal levels of p-T172-AMPK- α in comparison to control (WT) cells. Furthermore, AICAR-induced phosphorylation of the AMPK substrates ACC and RAPTOR was sub-optimal, specifically in the 1q21.1 Del containing line. The last observation could be explained by the fact that AMPK, as a multi protein complex, may be sensitive to imbalances of its components and that reduced availability of a regulatory β -isoform, as occurs here, could impact on AMPK activity more than its over-abundance. Interestingly, reduced AMPK activity in the skeletal muscle was observed in AMPK- β 2 knockout mice, associated with insensitivity to AICAR treatment (Dasgupta et al. 2012).

The multifaceted nature of AMPK role in brain function is of particular interest to the 1q21.1 phenotype, which most consistently includes some form of learning difficulty (Ronnett et al. 2009). Interestingly, the mouse AMPK- β 2 subunit is moderately expressed by most neurons, with the exception of the cerebellar Purkinje cells which express very high levels of AMPK- β 2 (Turnley et al. 1999). Previous studies showed that alternations of AMPK activity resulted in profound abnormalities of the central nervous system in AMPK- β 1 knockout mice (Dasgupta et al. 2009), which had a reduction of AMPK activity, whereas the consequences of AMPK activation remain controversial as some groups have shown that AMPK activation is neuroprotective while others show that AMPK overactivation is detrimental (Ronnett et al. 2009). The essential role of AMPK in brain function is further supported by its inhibition of the mTOR pathway (Kahn et al. 2005), which is required for learning and memory .

AMPK has also an established role in modulation of aspects of mitochondria function and mitochondrial biogenesis, and abnormal mitochondria function has been observed in a number of neurological and psychiatric disorders (Qi et al. 2010; Mattson et al. 2008). Evaluation of mitochondria function in 1q21.1 CNV carriers using mitochondria-specific dyes suggests mitochondria function is not grossly impaired in LBL's from 1q21.1 CNV carriers: it would be very interesting to know if this was the case in patient's neurons.

Our studies are the first to report that the function of two genes (*CDH1L* and *PRKAB2*) integral to 1q21.1 CNV are changed in patient lymphoblasts and that both genes are likely to be dosage sensitive. Both genes are expressed in multiple tissues, including brain (Dasgupta et al. 2009), which may explain the multi-systemic nature of the physical abnormalities, and the frequent involvement of learning difficulty albeit at a very variable levels. It remains uncertain as to the tissue-specific consequences of gene function changes in individuals with 1q21.1 CNV although AMPK is clearly involved in brain development and homeostasis.

The phenotypic variability for some CNVs has been traditionally explained by genetic and environmental factors (Iascone et al. 2002). In that respect it is of interest to note that *CHD1L* and *PRKAB2* have a role in sensing and responding to genomic (chromosomal structure) and metabolic (energy level) stress and therefore their dysfunction may result in a more severe phenotype in individuals, which experienced more adverse environmental conditions during development. Sequence changes of other genes from the 1q21.1 region as well as other genes across the genome that impair their function cannot be ruled out as a source of variability at this time and the new whole genome sequencing technologies will no doubt become useful in future investigations of their contribution to the development of an abnormal phenotype. My findings could prompt the use of AMPK activity modify therapies in animal models of this CNV. For example the use of AMPK *activators* in the case of *PRKAB2* deletion.

In conclusion, I provided evidence of changes in the function of a gene from 1q21.1 CNV in lymphoblasts from both deletion and duplication carriers. Deletions and duplications can have similar (e.g. *CHD1L*-associated DCC deficiency in 1q21.1 Del and Dup containing LBCs) as well as differing functional consequences (e.g. *PRKAB2*-associated less responsive AICAR-induced AMPK activity in 1q21.1 Del containing LBLs) depending on the genes and pathways involved. These results support the use of patient lymphoblasts for dissecting the functional sequelae of genes integral to CNVs in carrier cell lines, ultimately enhancing understanding of biological processes, which may contribute to the clinical phenotype.

Chapter Nine

*Discussion: Understanding at the molecular level
the clinical consequences of defective ATR
function in humans*

Discussion

This thesis provided novel insights on *how* defective ATR function can manifest in the specific set of clinical features that are observed in some forms of microcephalic primordial dwarfism (MPD).

A hypomorphic *ATR*-mutation causes a form of MPD classified as Seckel syndrome; which is linked to a defective ATR-dependent DNA damage response (O'Driscoll et al. 2003). Significantly, other forms of MPD have been associated with impaired ATR-dependent function, even when the causative mutation is not in *ATR per se*. This is observed for example in *PCNT*-mutated MOPDII (Griffith et al. 2008), and *ORC1*-mutated MGS. Collectively, this signifies that compromised ATR function is linked to profound growth delay, severe microcephaly with mental retardation, and skeletal abnormalities.

Atr depletion in mice results in replicative stress during embryogenesis, with accumulation of DNA damage and increased apoptosis (Murga et al. 2009). This could account for a considerable depletion of the stem cell pool, particularly during neurogenesis, as well as loss of total cellularity, which could underlie some of the clinical features seen in Seckel syndrome. However, the disproportionate impact on the skeletal tissue is not fully explained by this mechanism. Furthermore, recent studies have suggested an expansion of the ATR (and ATM) functional network beyond the DNA damage response (Matsuoka et al. 2007), which should prove of crucial interest for the understanding of all the signaling pathways involved. Therefore, and in order to investigate the genotype-to-phenotype relationship in MPD, I examined in particular whether ATR function is required specifically for skeletal development.

In Chapter 3 I showed that *ATR* inhibition did not compromise initiation of osteogenesis in MG-63 osteosarcoma cells, but there was some indication that maintenance of the differentiated status was not fully supported in *ATR* deficient cells. This could be an interesting observation in the context of the osteoporosis condition, defined as pathological loss of bone mass, which was observed after conditional *Atr* knockdown in adult mice (Ruzankina et al. 2007). Still on chapter 3, I presented findings derived from an optimized chondroinduction model system that allowed de-differentiation of MPD-derived fibroblastic lines into chondrocytes. Analysis of several markers of chondrogenic differentiation suggested that ATR-S

and PCNT-S patient cells are not able to efficiently form chondrocytes, when compared to control fibroblasts. These observations were confirmed by *ATR* and *PCNT* knockdown in control cells.

HIF-1 α promotes chondrocyte survival in the hypoxic fetal growth plate (Schipani 2006), and its expression is induced under *normoxic* conditions in my chondroinduction system. Significantly, both ATR-S and PCNT-S patient derived cells fail to adequately induce HIF-1 α , and VEGF expression (required for recruitment of blood vessels to the growth plate). This data suggests a serious problem in the regulation of the hypoxia response in ATR-function deficient cells, and altogether an impact of ATR depletion in several aspects of cartilage differentiation. Additionally, I provided evidence for the first time suggesting that defective ORC1 function, seen in MGS; as well as impaired IFT43, in the context of Sensenbrenner syndrome, are also associated with aberrant chondroinduction. Since the majority of the bones in the skeleton are formed using a cartilage template, a defect specifically in chondrogenesis would effectively result also in compromised bone formation and skeletal abnormalities. Interestingly, some forms of MPD, namely Meier-Gorlin syndrome, exhibit distinguishing defects in cartilaginous tissues, such as the absence of ear lobes, hypoplastic ears and small and/or absent kneecaps (Faqeih et al. 2005).

Importantly, all these phenotypes are disengaged from ATR's (as well as PCNT's and ORC1's) role during replication - since induction of chondro-differentiation is accompanied by exit from the cell cycle - and are therefore not associated with replicative stress.

My novel findings provide a cell-based model to help explain the origin of skeletal abnormalities in multiple MPD's, and establish a previously unsuspected role for ATR function in differentiation processes, specifically its involvement in skeletogenesis via chondrogenesis.

RTK signaling via the PI3K-AKT-mTOR pathway is essential for regulation of cell and organismal growth, and mutations in some components of this pathway can also result in growth retardation or dwarfism. Interestingly, numerous novel substrates for ATR phosphorylation in response to DNA damage are components of the insulin-AKT-mTOR network, although the biological significance of these interactions is yet unknown.

Affected insulin/IGF-dependent signaling in the context of ATR depletion has been insinuated in the literature. The humanized ATR-Seckel mouse has a suppressed growth hormone-IGF1 axis, and *acanthosis nigricans*, linked to insulin resistance, has been reported in Seckel syndrome patients. Furthermore, in Chapter 3 I showed that some crucial chondrogenic events are under PI3K/AKT regulation, and these are also being misregulated in ATR-S cells. Conversely, *Igf-1* and *Irs* mutant mice exhibit a severe growth phenotype (Kitamura et al. 2003).

Analysis of insulin-dependent PI3K-AKT-mTOR signaling in ATR-defective and PCNT-defective lines (Chapter 4) revealed deregulation of this important signal transduction pathway in MPD. Defects in this pathway may also underlie some of the clinical features observed in these patients. Interestingly, the two MPD lines exhibited a distinct pattern of deregulation/unresponsiveness throughout the PI3K-AKT-mTOR signal transduction, suggesting that different mechanisms could account for the defect in insulin response in ATR-S and PCNT-S.

In ATR-deficient cells, over-activation of the tuberous sclerosis complex (TSC1/TSC2), the principal negative regulator of mTORC1, results in unresponsive mTORC1-mediated phosphorylation of S6K. I also found evidence of endogenously elevated phosphorylation of S6K in ATR-S, which was not inhibited by short treatment with rapamycin under the conditions assessed, compared to control cells. Importantly, hyper or prolonged activation of S6K can result in inhibition of insulin-receptor mediated signaling, and has been associated with insulin resistance in type II diabetes (Shah et al. 2004). Although a mechanism for how ATR signal impairment can result in TSC2/TSC1 over-activation is not proposed here, it is noteworthy that TSC1 was identified as a direct substrate for ATR phosphorylation in response to DNA damage (Matsuoka et al. 2007). This suggests a previously unsuspected link between these two molecules, and pathways, whose biological significance is yet unknown. Interestingly, at least one study has shown that inhibition of the ATR-related ATM kinase results in decreased IGF-1 receptor expression (Peretz et al. 2001).

With regards to PCNT-S, insulin-dependent signaling is specifically compromised by failure to activate PI3K. Furthermore immunofluorescence studies in these cells revealed significantly reduced levels of PI3K. This indicates a serious defect impacting on an upstream event in this signaling pathway, and which could also affect other signaling cascades that are PI3K-signaling dependent. I confirmed a

direct interaction between PCNT and PI3K, by immunoprecipitation, and observed also reduced PI3K expression following *PCNT* depletion in an independent cell line. Interestingly, *PCNT*-mutated MPD has been specifically associated with *severe* insulin-resistance and diabetes (Huang-Doran et al. 2011). Decreased levels of PI3K could contribute to the insulin-resistance and diabetes phenotype in these patients.

Altogether, impaired insulin-mTOR signaling in ATR and PCNT-defective cells constitute a novel pathomechanism for ATR and PCNT-defective MPD, which could contribute to the severe dwarfism as well as some of the other phenotypes observed in these patients, namely insulin resistance, and skeletal defects.

Severe growth retardation, and microcephaly, the defining features of MPD, are also manifested at various levels in metabolic disorders of the mTOR pathway, such as Donohue syndrome (DS), which is caused by mutations in the insulin receptor (INSR). I analyzed cells derived from DS patients to further investigate the level of crosstalk and the functional relationship between the ATR-dependent DDR and mTOR pathway function (Chapter 5).

Surprisingly, *INSR*-mutant LBLs (and *INSR*-knockdown cells) are defective in the ATR-dependent activation of the G2/M checkpoint. This was also the case for *PTEN*-deficient Cowden's disease LBLs. PTEN has been shown to have a role in this checkpoint, whereby PTEN depletion results in cytoplasmic sequestration of CHK1, therefore impairing CHK1-mediated checkpoint activation (Puc et al. 2005). Importantly, loss of checkpoint proficiency could be a source of genomic instability in these cells, with implications for the high cancer predisposition observed in Cowden's disease and other PTEN-hamartoma diseases.

A novel mechanism for the regulation of the ATR-dependent G2/M checkpoint in INSR-defective cells was suggested here by my findings. I proposed that ATR/GSK-3 β signaling mediates an enhancement of the ATR/CHK1-dependent activation of the G2/M cell cycle checkpoint, acting downstream or parallel to CHK1 activation by ATR by directly phosphorylating CDC25A and targeting it for proteasomal degradation. Consequently, loss of the contributing effect of GSK-3 β down-regulation of CDC25A levels results in increased activation of the CDK1/CyclinB complex and premature entry into mitosis. Furthermore, this was dependent on AKT increased activation in DS cells, either due to lack of INSR-mediated feedback inhibition of the pathway, and/or via compensatory AKT phosphorylation from PDK1. I found AKT phosphorylation is induced in WT cells

following UV irradiation, which is translated in increased inhibition of its downstream target GSK-3 β . Notably, in DS cells this effect is enhanced. Inhibition of GSK-3 β (via siRNA, or use of specific inhibitors) replicated the checkpoint defect in control cells; and treatment with a specific AKT inhibitor rescued the checkpoint defect in *INSR*-defective cells.

Surprisingly, AKT inhibition also rescued the ATR-dependent checkpoint defect in PCNT-S cells, but not in ATR-S cells. This data further suggested that different mechanisms underlie the DDR defect in ATR and PCNT-defective SS/MOPDII cells; and is consistent with a defect in PCNT-S cells affecting a more upstream point in the insulin-PI3K-AKT cascade, likely the PI3-kinase itself, as suggested in Chapter 4.

AKT hyperactivation has been shown to override cell cycle checkpoints and promote cell proliferation in the presence of DNA damage (Xu et al. 2010; Xu et al. 2012). It is likely that the *level* of basal AKT activation is important in modulating this response. It is possible that the levels of AKT activation in DS were not sufficient to surpass the ATM-dependent DDR signal, which is activated in response to extensive DSB-inducing DNA damage.

A clearer understanding of how AKT is able to modulate the DDR could have broader implications, as AKT activation has been associated with enhanced proliferation and survival in tumor cells, and genomic instability, which could also derive from impaired checkpoint proficiency.

A novel cellular defect has been linked to the particular clinical presentation of microcephaly and growth retardation in a syndromal context: a newly described microcephalic disorder caused by mutations affecting the endosome-associated deubiquitinating enzyme, STAMBP. Microcephaly-capillary malformation syndrome (MIC-CAP) patients exhibit severe microcephaly, intractable seizures, post-natal growth retardation and extensive vascular malformations (Carter et al. 2011; Isidor et al. 2011; Mirzaa et al. 2011)

Functional analysis of cell lines derived from MIC-CAP individuals (Chapter 6) revealed elevated ubiquitin-conjugated protein aggregation, increased autophagosome formation, and elevated apoptosis induced by nutrient depletion. Elevated apoptosis is a recognized cause of microcephaly (Kuida et al. 1998; Schmitt et al. 2006; Silver et al. 2010; Cox et al. 2006). Additionally, autophagic clearance of

accumulated protein aggregates is an important mechanism disrupted in neuropathologies and neurodegenerative conditions such as Alzheimer's, Parkinson's and prion disease (Ventrucci et al. 2007). Therefore it is probable that these cellular phenotypes could cause progressive apoptosis in neuronal cells, and be important contributors to the microcephaly, mental retardation and seizure phenotype exhibited by MIC-CAP patients.

Furthermore, active and insensitive RAS-MAPK and PI3K-AKT pathway signaling were also observed in *STAMBP*-mutated LBLs. These phenotypes could contribute to the vascular defects and growth delay in MIC-CAP, and justify the inclusion of MIC-CAP in the class of disorders known as RASopathies. Hyperactive RAS signaling is a known instigator of oncogenic transformation. Although the cancer susceptibility in MIC-CAP is not known, it is likely that the increase in RAS-MAPK signaling is stable but discrete when compared to the more aggressive signal derived from oncogenic RAS mutations; and therefore more in keeping with the level of RAS activation in the other RASopathies. Nevertheless, increased tumor susceptibility is also a feature of most RASopathies, and therefore potentially of MIC-CAP as well.

Chronically activated signaling via internalized cell surface receptors, due to lack of *STAMBP* function, could provide a mechanism to explain the elevated activation of these signaling pathways.

Surprisingly, MIC-CAP patient LBLs also exhibit defective ATR-dependent G2/M checkpoint activation, which was not in this case mediated by elevated AKT signaling, as indicated by inhibitor studies. It is possible that defective DDR response may have a role in the development of microcephaly also in MIC-CAP. The brain abnormalities in *Stambp*^{-/-} mice are region-specific, with marked cerebral neuron loss and degradation of the CA1 hippocampal layer, with relative sparing of other CNS tissues such as the hippocampus (Busino et al. 2003; Suzuki et al. 2011; Ishii et al. 2001). In a recent study, conditional depletion of *Atr* throughout the mouse CNS revealed a relatively late and selective impact of ATR function during neurogenesis (Lee et al. 2012), involving only certain progenitor populations. The authors therefore refuted the suggestion that ATR's role in this process is the general prevention of replicative stress, but rather involves a regulated surveillance of genomic integrity in particular tissues and in a time-dependent manner. The mechanistic association between *STAMBP*-function and checkpoint activation

remains unclear, but it suggests a previously unknown route to cell cycle dysfunction. Importantly, a pool of STAMBP has been found to localize to the nucleus, suggesting additional and yet unknown functions for STAMBP at this site that could potentially be relevant for DNA repair pathways.

This study expands the list of known microcephalic disorders associated with defective ATR-dependent DDR; presents the first example of a human disorder caused by congenitally-defective endosome-associated DUB; and highlights an important functional interplay between RTK-mediated signal transduction and the ATR-dependent DDR, which could provide significant new insights into the pathophysiology of human microcephaly.

PI3K (*PIK3CA*; *PIK3R2*) and *AKT3* mutations were recently found in two related *megalencephaly* disorders (MCAP and MPPH). This suggests a key function of the PI3K-AKT-mTOR signaling pathway in the regulation of brain abnormalities, but also limb and vascular defects. Indeed components of this pathway have been implicated in human overgrowth syndromes, such as Proteus syndrome (caused by an activating *AKT1* mosaic mutation), the PTEN-hamartoma diseases, including Cowden's disease and autism with severe megalencephaly (Peng et al. 1997; Lindhurst et al. 2011; Cazales et al. 2005; Butler et al. 2005; Smith et al. 2009; Orrico et al. 2009).

Analysis of a panel of *PIK3CA*, *PIK3R2*, and *AKT3* mutated megalencephaly patient-derived LBLs (Chapter 7) established an association between brain overgrowth and increased activation of PI3K-AKT-mTOR signaling. Mutations in PI3K subunits (*PIK3CA*, *PIK3CB*, *PIK3CD*, *PIK3R1* and *PIK3R2*) have been observed in many human cancers (Cancer Genome Atlas Research Network 2008), but until now not in individuals with developmental disorders. Presently however, new mosaic gain-of-function mutations in *PIK3CA* have also been implicated in other distinct overgrowth conditions: hemimegalencephaly patients (also mutations in *mTOR* and *AKT3*) (Lee et al. 2012), mosaic overgrowth disorder with fibroadipose hyperplasia (Lindhurst et al. 2012), congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies (CLOVES) syndrome (Kurek et al. 2012) and macrodactyly (Rios et al. 2012).

Interestingly, analysis of megalencephaly patient LBLs revealed also an inefficient ATR-dependent G2/M checkpoint activation. Cowden's disease and other

disorders associated with *PTEN* deficiency, share clinical features with MCAP and MPPH, including brain overgrowth, and have also been associated with defects specifically in the ATR-dependent G2/M checkpoint (see Chapter 5). It is possible that the checkpoint defect observed in these particular disorders could be linked to increased AKT activation, which has been known to surpass cell cycle checkpoints and induce mitosis in the presence of types of DNA damage (Kandel et al. 2002). The data presented in this thesis with regards to megalencephaly may further support the importance of an efficient DNA damage response during normal brain development. Furthermore, it extends the list of human overgrowth syndromes associated with aberrant PI3K/AKT signaling, demonstrating that dysfunction of PI3K/AKT pathway signaling causes a constellation of brain, vascular and limb malformations. These findings combined with the development of inhibitors for PI3K, AKT or downstream targets such as mTORC1 could provide new therapeutic opportunities for megalencephaly as well as other developmental syndromes.

Curiously, both brain reduction as well as brain overgrowth have been here associated with increased signaling via the PI3K/AKT pathway (MIC-CAP *vs* MCAP/MPPH).

Syndromes with dramatic PI3K-AKT pathway hyperactivity are often associated with cell or organism overgrowth (e.g. Cowden's disease, tuberous sclerosis, Peutz-Jehgers syndromes). In contrast, disorders characterized by pure RAS-MAPK hyperactivity such as Noonan and Costello are associated with delayed development, short stature, and heart and vascular abnormalities. It is possible that the biological contribution of PI3K-AKT activation in MIC-CAP is counterbalanced by RAS-MAPK-dependent signaling, and importantly, by the elevated accumulation of ubiquitinated protein aggregates, defective ATR-dependent checkpoint activation, and resulting elevated apoptosis in *STAMBP*-deficient cells, which would be primary contributing factors to the clinical presentation of micro-(instead of macro)cephaly, growth retardation and vascular problems. Furthermore, a comparison between the *levels* of PI3K-AKT activation between microcephalic MIC-CAP and the megalencephaly disorders, might reveal another distinction between these disorders that could result in opposing phenotypes. Integration of all the inputs from the different intervening and interconnected signal transduction pathways, likely tips the phenotypic outcome in one direction *vs* another with respect to growth regulation.

ATR-dependent signaling is sensitive to haploinsufficiency for genes associated with ATR function (e.g. RPA, and ATR itself). This is seen in a set of haploinsufficient contiguous deletion disorders, such as Miller-Dieker lissencephaly and Williams-Beuren syndromes (O'Driscoll et al. 2007), which are also associated with microcephaly and growth retardation, among other distinctive features.

Copy Number Variation of DNA sequences (generally encompassing large regions >1Kb) are common occurrences that contribute to normal genomic variation. Additionally, these structural alterations in the human genome are commonly found in cancers (Stankiewicz et al. 2010; Colnaghi et al. 2011), but also in congenital Genomic Disorders, often accompanied by complex phenotypic traits (Colnaghi et al. 2011). Therefore the impact of CNV on dosage-sensitive pathways/processes, could also provide an important insight into the biological processes that contribute to particular clinical features.

1q21.1 Copy Number Variant (CNV) is associated with a highly variable phenotype. 1q21.1 CNV carriers exhibit learning problems/ intellectual disability (ID) of various degrees, congenital anomalies, dysmorphic features, making the clinical significance of this CNV difficult to evaluate.

In Chapter 8 I show that the 1q21.1 CNV can not only cause changes in the expression of one of the genes within the CNV, but also results in changes in their known function, as exemplified by impaired AMPK activation in the 1q21.1 Del LBLs. AMPK is involved in brain development and homeostasis and can impact on mTOR function. My findings could prompt the use of AMPK activity modify therapies in animal models of this CNV. For example the use of AMPK *activators* in the case of *PRKAB2* deletion. Furthermore, this study supports the use of patient lymphoblasts for dissecting the functional consequences of genes integral to CNVs in carrier cell lines, ultimately increasing understanding of biological processes which may contribute to the clinical phenotype.

The data provided in this thesis and ongoing research shows that the investigation of the basic mechanisms that regulate cell and organismal growth can benefit from studies of MPD and human overgrowth disorders. The re-evaluation of the roles of some of the MPD-causative genes at the cellular and developmental

levels was also proposed, exemplified by ATR's impact on specific differentiation processes (namely chondrogenesis) but also defects in fundamental replication proteins such as ORC1, which cause reduced growth and specific developmental abnormalities. Additionally, novel links were established between the DNA damage response and defects in such distinct proteins as the insulin receptor, and the endosome-associated deubiquitinase STAMBP, thereby implicating distinct cellular mechanisms and pathways in the regulation of these processes, with direct relevance to human health.

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